PANCREATIC PROGENITOR CELLS IN MICE

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Abstract

We generated a novel transgenic mouse expressing a tdTomato fluorophore, as well as a Strep/Flag-tagged version of Ptf1a from the native Ptf1a locus. I crossed this mouse line with the well-characterized Pdx1-GFP mouse, which enabled us to visualize and sort for cells of the "tip" and "trunk" progenitor domains of the mouse pancreas.

The main goal of our project was to identify previously unrecognized early transcriptional targets of Ptf1at and Pdx1. I isolated early E11.5 epithelial progenitors as well as later E13.5 tip and trunk progenitors using FACS and analyzed these populations via the GeneChip Mouse Gene 1.0 ST Array.

After comparison of microarray data between tip and trunk cells, differentially-expressed genes were identified, with a focus on transcription factors, with validation by in situ hybridization. Two transcription factors, Ascl2 and Lhx1, were initially identified that had no previously known role in pancreas development, and were shown by ISH to be expressed in the expected domain of the pancreas.

I performed prelimary functional studies on these two transcription factors, using lentiviral shRNAs for knock down in dorsal pancreatic bud culture.

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Chapter 1 – Introduction

Overview of Pancreas Development

The pancreas is an organ with both exocrine and endocrine functions. The exocrine pancreas produces and secretes digestive enzymes such as trypsin and carboxypeptidase, while the endocrine pancreas produces several different types of hormones including insulin, glucagon, somatostatin and ghrelin. The great majority of the pancreas, about 95%, is exocrine tissue, composed of acinar cells and ducts [1]. The acinar cells produce the digestive enzymes, which are secreted into the ducts and eventually into the small intestine. The endocrine cells are grouped into clusters called islets. Greater than 90% of the islet is composed of the insulin producing β-cells. The remainder of the islet is composed of α-cells, which produce glucagon; δ-cells, which produce somatostatin; PP cells, which produce pancreatic polypeptide; and ε-cells, which produce ghrelin [2].

The pancreas originates from cells that bud off of the endoderm. There are two pancreatic buds, dorsal and ventral, which fuse around E14 to form a single pancreas [3, 4] At the 7 Somite stage, which occurs around E8.25 in mice, the transcription factor Pdx1 is expressed in all pancreatic progenitor cells as well as posterior foregut progenitors [4]. Without expression of Pdx1, the pancreas fails to form. Ptf1a, also known as p48, is another essential transcription factor for pancreas development. Without Ptf1a, the pancreas fails to form properly, and some of the cells that were fated to form pancreas will instead follow an intestinal cell fate [5]. Ptf1a is expressed in all pancreatic progenitors starting around E9.5 in mouse embryos, and becomes progressively restricted during development.

Mouse pancreas development can be divided into three stages: the primary transition, the secondary transition, and the postnatal period. During the primary transition, which starts around E9.5, the embryonic pancreas buds out from the foregut endoderm and starts to express low levels carboxypeptidase, insulin and glucagon. Early in the primary transition, carboxypeptidase-expressing cells are capable of forming all three pancreatic lineages: acinar, ductal and endocrine. All epithelial cells during this stage express both of Pdx1 and Ptf1a. Around E12.5, the secondary transition begins. During the secondary transition, which is marked by a surge of endocrine and exocrine differentiation, the pancreas grows and branches, forming a network of inner "trunks" and outer "tips" [6, 7]. By E13.5, there are distinct tip and trunk progenitor cells. The tip progenitor cells express Ptf1a and lower levels of Pdx1; these cells will form the acinar cells. The trunk progenitor cells no longer express Ptf1a, but still express high levels of Pdx1; these cells will form the endocrine and ductal cells in the mature mouse pancreas. As the pancreas continues to mature, Ptf1a will eventually become completely restricted to the acinar cells and Pdx1 will be restricted to the endocrine cells [7–9].

The Exocrine Pancreas

Adapted from Exocrine Ontogenies: on the development of pancreatic acinar, ductal and centroacinar cells [10].

Metazoans have developed highly specialized exocrine cell types dedicated to the synthesis and secretion of proteolytic and other digestive enzymes. Examples across multiple phyla include zymogen gland cells in hydra endoderm [11], F-cells in the crustacean hepatopancreas [12], hatching gland cells in teleost and amphibian embryos [13, 14], and the acinar cells of the vertebrate pancreas. In most vertebrates, pancreatic

acinar cells develop and function in close spatial proximity to their endocrine counterparts, and are presumed to be derived from a common multi-lineage progenitor cell (MPC).

Driven by the need to develop treatments for diabetes, much of the research in pancreatic developmental biology has historically focused on the endocrine compartment. Recently, studies focusing on mechanisms of exocrine pancreas development have become increasingly common. These studies reflect the important role of acinar and ductal cells in pancreatic disease, as well as an increasing awareness that adult exocrine cell types might serve as effective sources for beta cell neogenesis [15, 16]. These studies have also demonstrated that endocrine and exocrine ontogenies are highly intertwined, and further suggested an unanticipated plasticity between lineages. In addition, emerging evidence suggests that the islet and ductal lineages share a common immediate progenitor, and are more closely related than the ductal and acinar lineages. Given this interplay, it may be somewhat disingenuous to consider exocrine pancreas development as an isolated topic. Nevertheless, in this review we summarize current knowledge regarding development of the exocrine pancreas, including the specification, differentiation and function of acinar, centroacinar and ductal cell types. In its focus on exocrine development, this review neglects many other areas of pancreatic developmental biology, including foregut patterning, early morphogenesis, endocrine differentiation and developmental plasticity. Outstanding summaries of these topics can be found in a number of recent reviews, including several published in the current issue [17–23]. While this effort clearly emphasizes principles generated from studies of pancreas development

in the mouse, where appropriate we also attempt to incorporate relevant observations from other vertebrate species.

Cell types of the exocrine pancreas

Pancreatic Acinar Cells—Acinar cells in the vertebrate pancreas are frequently considered to be *the* paradigmatic polarized secretory cell; they were used in the Nobel Prize-winning initial demonstration of the vectorial sequence of protein trafficking from endoplasmic reticulum (RER), to Golgi, to condensing vacuole, to secretory granule [24]. Pancreatic acinar cells are pyramidal in shape, and quite large, reaching up to 30 microns in apical-to-basal height. The acinar cell's extreme dedication to the synthesis and secretion of digestive zymogens is demonstrated by a remarkably dense accumulation of rough endoplasmic reticulum, as well as an apical cytoplasm laden with secretory granules. This high degree of specialization is also evident on a molecular level, where a remarkable fraction of total acinar cell mRNA is devoted to transcripts encoding digestive zymogens [25].

In addition to multiple well known proteolytic enzymes, pancreatic acinar cells synthesize and secrete glycoside hydrolases such as Amylase, as well as ribonucleases, lipases and phospholipases [26, 27]. Among these enzymes, Amylase, Trypsin(ogen), Carboxypeptidase A (CPA), and Elastase are most frequently employed as acinar cell-specific markers. Other commonly utilized acinar cell markers include the plant lectin Peanut Agglutinin (PNA) [28] and the transcription factors Ptf1a, Mist1 and Rbpjl [29, 30]. Acinar cell-specific gene expression is frequently driven by the heterotrimeric PTF1 transcriptional complex, which binds to tandem E- and TC-box elements found in enhancer/promoter elements of many zymogen genes [31]. While the expression levels

of different digestive zymogens are frequently assumed to co-vary, there is evidence of differential regulation of different zymogen classes, as well as non-synchronous activation of zymogen gene expression during acinar cell differentiation [25, 32, 33].

Centroacinar Cells—The acinus consists of an organized cluster of acinar cells which secrete digestive enzymes into a central lumen, from which the enzymes flow into ducts. Perhaps the most enigmatic of all pancreatic cell types, the centroacinar cell lies at the junction of the secretory acinus and its associated terminal ductal epithelium. These cells are variably depicted as an extension of the most terminal ductal epithelium as it invaginates into the secretory acinus [34], or alternatively as providing a fenestrated "cap" to the apical surface of acinar cells [35]. At this point, it remains uncertain whether centroacinar and terminal duct cells represent two different cell types or are functionally equivalent, and the possibility exists that multiple cell types may be located in a centroacinar position. In contrast to the much larger pancreatic acinar cells, the main body of centroacinar cells is typically less than 10 microns in diameter, with minimal cytoplasm and a high nuclear-to-cytoplasmic ratio. Work in both mammalian systems and zebrafish has suggested that these cells also extend long cytoplasmic processes, providing apparent contact with other centroacinar cells as well as adjacent acinar and islet cell types [34, 36]. While this unique feature is quite distinctive, the functional significance of these extensions remains unknown.

In addition to their unique location and morphology, many studies have also called attention to centroacinar cells as a candidate multipotent progenitor cell in adult pancreas [37–41]. Centroacinar cells have been shown to rapidly proliferate following either partial pancreatectomy [39, 42], streptozotocin induced destruction of the insulin-

producing β-cells [38, 43], and acute or chronic administration of caerulein [40]. As discussed more extensively below, our group and others have also identified centroacinar and terminal duct cells as the exclusive domain of active Notch-signaling in adult mouse and zebrafish pancreas; this is evidenced by the expression of Hes1, a Notch-target gene, or by the activation of a Notch-responsive fluorescent reporter in these cells [37]. In addition to ongoing Notch-pathway activation, at least some centroacinar cells also express Sox9, another marker of progenitor cells in the developing pancreas [38]. We have also demonstrated that a centroacinar cell population is characterized by high-level ALDH1 enzymatic activity. Furthermore, these cells display a unique *in vitro* progenitor capacity, including a markedly heightened ability to form pancreatospheres in suspension culture, as well as a unique ability to contribute to embryonic exocrine and endocrine lineages. Nevertheless, the ability of ALDH1-expressing centroacinar cells to act as *in vivo* progenitors has never been documented, and while the degree of overlap between the ALDH1, Sox9 and Hes1 lineages remains to be established, *in vivo* analyses of the adult Sox9 and Hes1 lineages have failed to detect multilineage progenitor activity [44, 45].

Developmentally, the origin of centroacinar cells has not been clearly established, in part due to a paucity of centroacinar cell-specific markers, as well as an incomplete understanding of possible cellular heterogeneity among cells residing in the centroacinar position. In zebrafish, adult centroacinar cells arise from progenitor cells within the larval pancreatic duct [46]. In the mouse, rare cells expressing Sox9 along with markers of both nascent ducts (e.g. Nkx6.1) and nascent acini (e.g. Ptf1a) have been identified at E14.5 [47]. As discussed below, these cells may represent an early manifestation of the centroacinar lineage.

Ductal Epithelial Cells—The adult pancreatic ductal epithelial tree serves two critical physiologic functions: first, to secrete bicarbonate-rich fluid to dilute and pH-optimize the protein concentrate secreted by acinar cells; and second, to convey this mix to the intestinal lumen. Ducts are typically classified by size and position within the ductal epithelial tree, with the most terminal/intercalated ducts draining into intralobular ducts, followed by interlobular ducts, and finally the main pancreatic duct, which drains into the intestine [48]. Classical conceptions of ductal morphogenesis have typically invoked a direct extension of the gut lumen into an ever more highly branched epithelial tree. However, recent highly detailed examinations of duct morphogenesis [49–51] suggest a more complex mechanism. This process involves initial epithelial stratification and the formation of multiple small lumens that subsequently remodel through changes in cell shape and position to ultimately fuse and form a ramifying, single-lumen ductal system.

Classical markers used to label and identify ductal epithelium include the functional markers Carbonic Anhydrase II, Mucin 1 (also known to label acinar cells), Cystic Fibrosis Transmembrane Receptor (CFTR), and various Cytokeratins (many of which display species-specific expression). Murine pancreatic ducts are also selectively marked by expression of CD133 and Osteopontin [47, 52, 53], as well as binding by the *Dolichos biflorus* (DBA) and *Wisteria fluoribunda* (WFA) plant lectins [28, 54]. Although many studies continue to consider the ductal epithelial tree as an essentially uniform structure along its peripheral-to-central axis, this likely represents a drastic oversimplification, and the detailed characterization of inter-segment heterogeneity represents an important challenge for the field. Considerable evidence already exists

supporting such segment-specific identities. Morphologically, the most terminal ductal epithelial cells exhibit a somewhat flattened or squamous shape and minimal cytoplasm, while more central stereotypical ductal epithelial cells are cuboidal and rich in mitochondria. At the level of protein expression, there also exists extensive evidence of selective marker expression along different segments of the ductal epithelial tree, including primarily peripheral expression of CFTR, Muc1, and Aquaporins 1 and 5 [55, 56]. In addition, different "duct-specific" transcription factors, such as Hnf1β, Hnf6 and Sox9, are expressed heterogeneously throughout the ductal epithelial tree (Figure 1.1); the significance of this heterogeneity has not yet been explored. Evidence also suggests that the normal morphogenesis of different segments of the ductal epithelial tree may be dependent upon different transcriptional programs. Mice lacking the transcription factor Hnf6 have normal terminal/intercalated ducts but exhibit cystic dilation of inter- and intra-lobular ducts [57]. Many additional aspects of pancreatic ductal biology have been nicely summarized in a detailed review [48].

In addition to the ductal epithelial segments discussed above, an important paper recently called attention to a previously noted but neglected ductal epithelial compartment referred to as the pancreatic duct gland; a compartment that had been previously noted, but which has been neglected in more recent work [58–61]. These glands are comprised of blind outpouches of the main pancreatic duct, and cells within these glands show a unique columnar morphology and express genes not found in normal cuboidal duct cells, including Shh, Pdx1, Hes1 and gastric-type mucins. In response to chronic epithelial injury, these glands undergo selective expansion and may contribute to formation of mucinous metaplasia and pancreatic intraepithelial neoplasia [58].

Regulation of exocrine differentiation by soluble morphogens

In mammals, the pancreas forms from separate dorsal and ventral pancreatic buds. The buds arise, proliferate and ramify within the adjacent dorsal and ventral pancreatic mesenchyme. Historically, initial insights into the regulation of exocrine pancreatic development focused on the influence of mesenchyme-derived soluble morphogens, including their influences on endocrine vs. exocrine lineage selection. The first evidence that mesenchymal tissue was required for the normal development of the pancreatic epithelium was provided by studies involving in vitro culture of microdissected E11.5 dorsal pancreatic buds [62]. Associated dorsal mesenchyme was demonstrated to be required for normal growth of the epithelial bud, as well as the development of exocrine acini, but not the expression of endocrine genes [62, 63]. These studies defined endocrine differentiation as the effective "default path" for isolated pancreatic epithelium [64]. Furthermore, these early experiments demonstrated that while mesenchymal signals were required for normal bud growth and eventual exocrine differentiation, subsequent to E10.5 the mesenchymal influence was permissive rather than instructive, with non-pancreatic mesenchyme from other segments of the foregut equally able to support pancreatic epithelial growth.

It is now apparent that these results reflect the fact that mesenchymal tissues associated with budding foregut derivatives frequently express a common set of soluble morphogens. For example, FGF10, produced by the dorsal pancreatic mesenchyme [65], is also expressed in mesenchymal tissues associated with the nascent lung buds [65, 66]. Mesenchyme-derived FGF family members indeed play a critical role in exocrine pancreatic development [67–69]. Specifically, FGFR-2 IIIb and its ligands FGF-1, FGF-

7, and FGF-10 are expressed throughout pancreatic development and promote the growth, morphogenesis, and differentiation of exocrine cells [67]. Subsequent studies have demonstrated that a significant component of the FGF10 influence involves the activation of epithelial Notch-signaling, thereby maintaining a pool of undifferentiated progenitor cells capable of supporting ongoing epithelial growth [68, 70, 71]. In addition to allowing progenitor cells to avoid early default endocrine differentiation, and thereby promoting later exocrine fates, the delay in progenitor differentiation induced by FGF10 effectively increases the window during which progenitor cells activate Ngn3 and contribute to the endocrine lineage. In so doing, FGF10 also effectively increases the ultimate number of endocrine cells [65, 72].

Other soluble morphogens capable of influencing exocrine differentiation include EGF, which promotes proliferative growth at the expense of both exocrine and endocrine differentiation [73], and members of the TGF-β/BMP superfamily. One such mesenchyme-derived factor, the TGF-β antagonist Follistatin, was found to mimic both inductive and repressive effects of the mesenchyme, promoting development of exocrine tissue while limiting endocrine differentiation [74]. Conversely, the addition of exogenous TGF-β to isolated pancreatic epithelium was found to promote endocrine at the expense of exocrine differentiation [75], and transgenic expression of a dominant negative TGF-β type II receptor was found to promote expansion of exocrine cells [76]. Together, these data suggest that pancreatic mesenchyme limits default endocrine differentiation through the combined effects of FGF signaling activation and TGF-β inactivation within the developing pancreatic epithelium.

While most studies examining the effect of soluble morphogens on pancreatic exocrine development invoke a mesenchyme-to-epithelium signaling paradigm, additional studies have identified autonomous mesenchymal signaling pathways that exert potent influences on the development of associated pancreatic epithelium. For example, analysis of phospho-Smads 1, 5 and 8 have suggested selective activation of BMP signaling in E11.5 mouse pancreatic mesenchyme, as well as in chick pancreatic mesenchyme at a similar developmental stage [77]. In the chick, this was associated with mesenchymal expression of BMP4 and BMP7, suggesting a mesenchyme-autonomous BMP signaling network. In both mouse and chick, inhibition of mesenchymal signaling following electroporation of the BMP antagonist Noggin resulted in pancreatic epithelial hypoplasia, reduced branching, excessive endocrine differentiation and impaired exocrine differentiation. Electroporation of a dominant negative Alk3 receptor into E11.5 mouse pancreatic mesenchyme effectively phenocopied the effect of Noggin [77]. These studies suggest that TGF-β type II receptor signaling in the epithelium and Alk3 receptor signaling in the mesenchyme exert opposing effects on exocrine lineage commitment and/or differentiation.

While many soluble morphogens appear to modulate competing progenitor commitment to the endocrine and exocrine lineages, recent studies suggest that multipotent pancreatic progenitor cells (MPCs) are allocated to spatially distinct trunk and tip domains, with trunk progenitors giving rise to primarily endocrine and ductal cells, and tip progenitors giving rise to primarily acinar cells (this concept is discussed in detail in the following section). It is, therefore, not surprising that specific soluble morphogens can also influence the relative allocation of progenitor cells to ductal and

acinar fates. Indeed, this influence appears to be mediated by retinoid signaling, which has been shown to promote ductal differentiation through up-regulation of mesenchymal laminin-1 [78–80]. Specifically, 9-*cis* retinoic acid (9cRA) inhibits acinar differentiation in the developing pancreas, in favor of ducts, and 9cRA does not induce ductal differentiation in the absence of mesenchyme or following inhibition of laminin signaling [79]. Another mesenchyme-derived soluble factor selectively promoting pancreatic ductal proliferation and differentiation is epimorphin (syntaxin 2), which is also known to induce epithelial branching in a variety of tissues [81, 82].

Recently, through the use of a mesenchymal-specific Nkx3.2 (Bapx1):Cre driver, the mesenchymal contribution to pancreas development was probed *in vivo* [83]. Using this Cre driver to accomplish selective ablation of the pancreatic mesenchyme using either Cre-activated Diphtheria toxin or Diphtheria toxin receptor alleles, this study demonstrated that mesenchymal cells are required to support normal pancreatic growth and branching at both early and late developmental stages. This effect was conveyed by promoting the proliferation of both differentiated and undifferentiated cell types, with minimal direct effects on endocrine and exocrine differentiation. Furthermore, mesenchyme-specific deletion of β-catenin largely phenocopied the effect of Diphtheria toxin-mediated mesenchymal ablation, implicating β-catenin as an essential mediator of mesenchymal expansion and survival [83].

While many of the influences of the pancreatic mesenchyme on pancreatic exocrine differentiation are presumed to emanate from mesenchymal fibroblasts, endothelial cells are also known to be potent modulators of both early and late pancreatic development [54, 84–87]. With respect to exocrine differentiation, recent studies have

demonstrated that vascular endothelial cells normally associate with ductal and islet progenitors in the trunk domain of the pancreatic epithelial bud, and specifically inhibit allocation of MPCs to the tip domain, thereby inhibiting acinar cell differentiation [84, 86]

Progenitor commitment to endocrine and exocrine fates: transcription factor topology in trunks and tips

Ultimately, the multiple soluble morphogens directing MPCs to adopt endocrine and exocrine cell fates convey their effects through altered expression and/or activity of lineage-specifying transcription factors. As in the case of progressive endocrine differentiation, the differentiation of exocrine pancreatic cell types requires specific temporal and spatial cascades of transcription factor activation. In the case of acinar cell differentiation, the basic helix-loop-helix transcription factor Ptf1a is required not only for the commitment of progenitor cells to an acinar cell fate, but also for maintenance of acinar cell differentiation in adult pancreas. In this regard, the role played by Ptf1a differs from the role played by the bHLH transcription factor Ngn3, which is required for initiation but not maintenance of endocrine differentiation. As discussed below, this difference is reflected in the contrasting manner in which these two bHLH proteins interact with the Notch-signaling pathway.

Over the past 15 years, it has become apparent that frequently reported reciprocal changes in endocrine vs. exocrine cell fates reflect the fact that all pancreatic epithelial cells types share a common origin from MPCs. These progenitors are defined by a unique gene expression signature involving at least partially overlapping expression of Pdx1, Ptf1a, Sox9, Hes1, Hnf1β, Nkx6.1 and Nkx6.2 [7, 8, 45, 47, 88–90]. While the common origin of both endocrine and exocrine cell types from pools of progenitor cells expressing

these transcription factors has been indisputably established by Cre/lox-based lineage tracing strategies [5, 45, 47, 89, 91], it should be stressed that unlike imaging- and transplantation-based lineage-tracing techniques often used in invertebrates, Cre/lox-based lineage tracing in mice and other vertebrates establishes the lineage of collective and potentially heterogeneous cell populations rather than individual progenitor cells. However, direct evidence of a specific MPC cell type contributing to both endocrine and exocrine lineages has been confirmed by clonal analysis of developing pancreatic epithelium. Single-cell labeling of E11.5 murine dorsal pancreatic buds using limiting dilutions of replication-incompetent retrovirus leads to subsequent clusters of cells with both endocrine and exocrine components [92]. ERT2 allows for temporal control of Cre-activity and more recent studies that used limiting dilutions of tamoxifen to activate cell type-specific CreER lineage labels in a highly mosaic manner have generated similar results [7, 45]. For the Hes1 lineage, low density and presumably clonal activation of a Hes1:CreERT2 lineage marker on E9.5 leads to expanded clusters of labeled islet, acinar and ductal cells at E17.5, indicating apparent clonal derivation [45]. Similar observations have been made following clonal activation of a Carboxypeptidase A lineage label at E11.5 [7].

While each of the above transcription factors appears to play a role in the specification or maintenance of the pancreatic progenitor pool, the commitment of MPCs to either the endocrine or exocrine lineages involves the progressive restriction of their initially overlapping patterns of expression. Spatially, this involves the progressive organization and patterning of the developing pancreas into distinct central and peripheral fields. In the setting of ongoing branching morphogenesis, these become resolved into

central “trunks” and peripheral “tips”; with trunk domains becoming progressively restricted to islet and ductal fates, and tip domains becoming progressively restricted to the acinar lineage [7, 88, 89]. Thus the progressive restriction of MPC fates does not respect a simple endocrine vs. exocrine paradigm, but instead involves selection between competing islet/ductal and acinar cell fates. In this discussion, developmental timepoints refer primarily to events in the dorsal pancreatic bud and to the onset of detectable protein expression or the timing of tamoxifen administration to activate lineage-specific CreER labeling.

Between E9.5 and E10.5, Pdx1, Ptf1a and Sox9 are co-expressed in the vast majority of MPCs [38, 90]. By E10.5, most Pdx1+, Ptf1+, Sox9+ MPCs also express Hes1 and Nkx6.1 [88, 93] Between E11.5 and E12.5, the stratified pancreatic buds begin to organize into a branched epithelial tree. By E11.5, the majority of cells have activated expression of Hnf1β and CPA, in combination with ongoing expression of Pdx1, Ptf1a, Sox9, Hes1, Nkx6.1, and Nkx6.2. At this stage, Cre/lox-based lineage tracing confirms that MPCs expressing Pdx1, Sox9, Hnf1β, Hes1 and CPA/Ptf1a remain tri-potent, effectively contributing to the future islet, ductal and acinar lineages [7, 45, 47, 89, 91].

By E12.5, overlapping expression of these transcription factors begins to diminish, and the bud becomes spatially resolved into trunk and tip domains. As this occurs, Ptf1a and CPA become progressively restricted to peripheral tips of the epithelial branches, while Hnf1β, Nkx6.1 and Nkx6.2 become progressively restricted to trunks [7, 88, 89]. In contrast, at E12.5, Pdx1 and Sox9 continue to be broadly expressed. Examples of trunk- and tip-restricted transcription factor expression are provided in Figure 1.2. Notably, the appearance of cells expressing only trunk- or tip-associated

genes occurs even before the onset of branching morphogenesis. For example, scattered cells expressing either Nkx6.1 or Ptf1a (but not both) can be detected as early E10.5 [88]; whether or not these cells are already lineage-restricted and subsequently spatially sort into appropriate trunk and tip domains, or remain plastic and modify trunk and tip gene expression based on location, remains unknown.

Associated with the establishment of discrete Ptf1a+, CPA+, Hnf1β-, Nkx6.1-, Nkx6.2-, Sox9+ tip domains and Ptf1a-, CPA-, Hnf1β+, Nkx6.1+, Nkx6.2+, Sox9+ trunk domains, MPCs become progressively lineage restricted. Studies using a tamoxifen-inducible Hnf1β:CreER allele have revealed that E11.5 and E12.5 Hnf1β+ progenitor cells contribute to the islet, ductal and acinar lineages, although the proportion of acinar cells originating from Hnf1β+ progenitors is measurably decreased following tamoxifen administration at E12.5 compared to E11.5 [89]. Conversely, lineage labeling using a CPA:CreER has demonstrated that E10.5, E11.5 and E12.5 CPA+ progenitors contribute to islet, ductal and acinar cells, but CPA+ cells labeled by tamoxifen injection on E13.5 contribute exclusively to the acinar lineage. Together, these studies demonstrate the progressive restriction of trunk progenitors to the islet and ductal fates, and a corresponding restriction of tip progenitors to the acinar lineage. In contrast, the pool of progenitor cells expressing Sox9 remains tri-potent up until birth, reflecting ongoing expression not only in trunk progenitors but also in a subset of cells at the junction of the tip and trunk domains [38, 44, 47]. In this regard, detailed studies of the tip/trunk junction at E14.5 have demonstrated a tip population of differentiating acinar cells expressing Ptf1a but not Hnf1β or Sox9, a trunk population expressing Hnf1β and Sox9, but not Ptf1a, and an intermediate population expressing Ptf1a and Sox9 but not Hnf1β

[88]. A subset of these Ptf1a+, Sox9+ cells also appear to express Hes1 [38]. As discussed below, these cells may represent the initial manifestation of the centroacinar lineage. In adult mouse and human pancreas, Sox9 expression is restricted to centroacinar and terminal duct cells, with negligible ongoing contribution to the endocrine lineage in the absence of injury [44, 47]. Following the induction of chronic pancreatitis by way of pancreatic duct ligation, Sox9+ centroacinar and terminal duct cells are capable of generating duct-associated cells expressing Ngn3, but these cells fail to complete an endocrine differentiation program [47]. While conflicting data exist regarding the normal contribution of adult Sox9+ cells to the exocrine lineage [44, 47], demonstration of a postnatal contribution of the adult Sox9+ cells to the acinar lineage appears to require activation of a Sox9:CreER lineage label with very high doses of tamoxifen. This raises the possibility of leaky Cre activity in acinar cells themselves, even in the absence of Sox9 protein expression.

While these studies have clearly defined the progressive fates of tip and trunk progenitor cells, a detailed mechanistic understanding of how each of these transcription factors contributes to the determination of acinar, ductal and centroacinar fates remains incomplete. Nevertheless, certain functional interactions have been clearly established. In particular, recent studies have demonstrated specific and opposing roles for Nkx6.1/6.2 and Ptf1a in promoting trunk vs. tip cell fates. Reflecting their initial co-expression but subsequent partitioning to trunk and tip domains [8, 88], recent functional studies have demonstrated antagonistic interaction between these factors. Specifically, the Nkx6 factors, which by E12.5 are largely restricted to trunk progenitors, have been demonstrated to be both required and sufficient to promote endocrine and antagonize

acinar cell fate. Tip-restricted Ptf1a exerts the opposite influence [88]. Combined deletion of Nkx6.1 and Nkx6.2 results in extension of Ptf1a expression into the E12.5 trunk region, coupled with an increase in the number of Amylase-positive cells and a corresponding decrease in the number of cells expressing Ngn3. In contrast, forced expression of either Nkx6.1 or Nkx6.2 throughout the pancreatic epithelium promotes endocrine differentiation, while repressing the acinar cell fate. Notably, the number of ductal epithelial cells is unaffected by ectopic Nkx6 factor expression, reflecting the shared origin of islet and ductal cells from the trunk domain [88].

Notch signaling negatively regulates exocrine differentiation

Superimposed on the resolution of the branching epithelial tree into central trunk and peripheral tip domains is the influence of the Notch-signaling pathway, known to be a critical regulator of both endocrine and exocrine differentiation in both mouse and zebrafish pancreas [36, 45, 46, 71, 94–101]. As described above, Notch pathway activation (as imperfectly assessed by Hes1 expression) appears to be widespread in early MPCs [45, 93, 97], and subsequently becomes progressively silenced coincident with the onset at E13.5 of burst of endocrine differentiation known as the secondary transition. Specifically, Hes1 expression is lost in endocrine progenitors as they activate Ngn3 and delaminate from trunk epithelium [102], as well as in Ptf1a- and CPA-expressing tip progenitors as they begin to initiate acinar cell differentiation [45, 97]. By E14.5, Hes1 expression is largely confined to Ngn3-negative, CPA-negative trunk epithelium. This expression likely includes cells in the emerging centroacinar compartment, and by birth Hes1 expression is entirely restricted to centroacinar and terminal duct cells, most of which also express Sox9 [38, 41, 45, 97]. Centroacinar localization of cells with active

Notch-signaling has also been observed in developing and adult zebrafish pancreas, and evidence suggests that these cells may also function as MPCs [36, 46].

This progressive restriction of Notch-signaling is associated with progressive restriction in the fate of the Hes1 lineage [45]. When labeled by tamoxifen injection at either E9.5 or E11.5, Hes1+ MPCs are tri-potent, contributing to the islet, ductal and acinar lineages. However, at the onset of the secondary transition at E13.5, these cells begin to progressively lose their potential to contribute to the endocrine lineage, with tamoxifen injection on E15.5 failing to label future alpha- or beta-cells. These data suggest that, during the secondary transition, the emergence of Ngn3+ cells from Hes1-expressing progenitors in the trunk domain occurs during a discrete temporal window between E13.5 and E15.5. In contrast, Hes1+ trunk cells, which become progressively restricted to the terminal ductal and centroacinar positions, continue to contribute to the ductal and acinar cell lineages throughout development, while serving as apparent lineage-restricted duct cell progenitors after birth [45]. Thus in addition to cells expressing Pdx1 and Sox9, Hes1+ centroacinar cells, perhaps related to the Ptf1a+, Sox9+, Hnf1β-negative cell population described on E14.5 [88], may represent the only other progenitor cell type capable of crossing the trunk/tip divide.

Functionally, Notch appears to effectively block both endocrine and exocrine differentiation [97–101]. In the case of endocrine differentiation, this occurs by direct repression of Ngn3 expression [99, 101–103], while in the case of exocrine differentiation, it appears to occur through selective inhibition of Ptf1a's late induction of acinar cell-specific gene expression [97, 104]. In contrast, the early pancreatic specification and morphogenetic influences of Ptf1a are permitted in the context of

activated Notch-signaling, and Ptf1a itself appears to play a role in early Notch pathway activation, through induction of Dll1 expression [93]. With respect to regulation of ductal differentiation, several studies have demonstrated that Notch promotes embryonic MPC's to adopt a trunk progenitor phenotype [88] and ultimately a ductal fate [45, 105]. Thus, the influence of Notch on pancreatic development may transcend a simple inhibitory effect on endocrine and acinar cell differentiation, and include an effect on lineage selection mediated through an influence on trunk/tip patterning. These concepts are presented schematically in Figure 1.3.

In adult pancreas, continued Notch activation in centroacinar cells appears to be required for ongoing contribution of these cells to the ductal lineage; selective inactivation of Notch-signaling in Hes1+ centroacinar cells leads to their rapid differentiation into acinar cells, eliminating their ability to serve as adult ductal progenitors [94]. The pro-ductal influence of Notch may be suggested by evidence of abnormal Notch pathway activation in the majority of human pancreatic ductal cancers [37].

Induction of "terminal" exocrine differentiation

While little is known regarding transcriptional regulation of ductal and centroacinar differentiation, the functions of specific transcription factors in islet and acinar differentiation have been well characterized. Associated with the onset of the secondary transition on or about E13.5, endocrine differentiation is initiated by loss of Hes1 and activation of Ngn3 in cells derived from trunk progenitors, followed by the activation of additional endocrine-specific transcription factors and the generation of individual islet cell types [22].

Similarly, acinar cell differentiation is initiated within the tip domain, characterized by ongoing expression of Ptf1a and additional activation of the mammalian Supressor of Hairless homologue Rbpjl [31, 106]. Co-expression of Ptf1a and Rbpjl appears to activate a self-reinforcing gene expression module, effectively locking cells into a fixed state of acinar cell differentiation [106]. However, even this state of apparent terminal differentiation can be reversed by either reactivation of Notch-signaling [37, 107] or by reprogramming with combinations of endocrine-specific transcription factors [16]. Another transcription factor required for generation of the mature acinar phenotype is the bHLH transcription factor Mist1. Expression of Mist1 appears to be a common feature of many differentiated exocrine cell types, including parotid acinar cells, gastric chief cells, and cells of the zebrafish hatching gland [108–110]. In pancreatic acinar cells, Mist1 is required for establishment of the mature secretory phenotype and further acts to limit the proliferation of mature acinar cells [30, 109, 111, 112]. Differentiating and differentiated acinar cells also express the nuclear receptor LRH1/Nr5a2 [113], which has been shown to both physically interact with Ptf1a and independently activate expression of genes encoding digestive zymogens and other acinar cell-specific genes. Conditional deletion of LRH1 in adult pancreatic tissue results in decreased expression of several acinar cell-specific genes, as well as altered concentrations of acinar cell proteases and lipases in secreted pancreatic fluid. In addition, LRH1-deficient mice displayed impaired secretagogue-induced pancreatic fluid secretion. This raises the possibility of an additional influence of LRH1 on adult pancreatic duct cell function, although it remains unknown whether or not this represents a duct cell autonomous effect [113].

As discussed above, multiple transcription factors appear to contribute to patterning of the central trunk domain from which ductal epithelial cells are subsequently derived. However, at present, no specific transcription factors have been implicated in the initiation and maintenance of duct-specific gene expression, although Prox1, Hnf1β and Hnf6 likely play instructive roles. In the normal pancreas, Prox1 is widely expressed at E13.5, but is low or absent in acinar cells in the adult [114, 115]. In a conditional knockout of Prox1, there is relatively normal expression of both Hnf6 and Hnf1β, while Prox1 expression is decreased in the Hnf6 deficient mouse, suggesting that Hnf6 is upstream of Prox1 [115, 116]. Prox1-deficient mice are viable, but have dilated ductal lumens and progressively decreasing amounts of acinar tissue [115], suggesting an possible role for Prox1 in maintaining normal duct function.

In the absence of identified positive regulators of ductal differentiation, it is tempting to consider that ductal epithelial cells represent the progeny of "left behind" trunk progenitors that have avoided an endocrine fate by virtue of active Notch-signaling and failure to activate high levels of Ngn3. While evidence exists supporting this hypothesis [105, 117, 118], it remains likely that additional positive regulators of ductal differentiation remain to be identified.

Acknowledgements

The authors wish to thanks Charles Murtaugh for helpful discussions. This work was supported by the Chicago Diabetes Project and NIH grant DK61215 (to SDL).

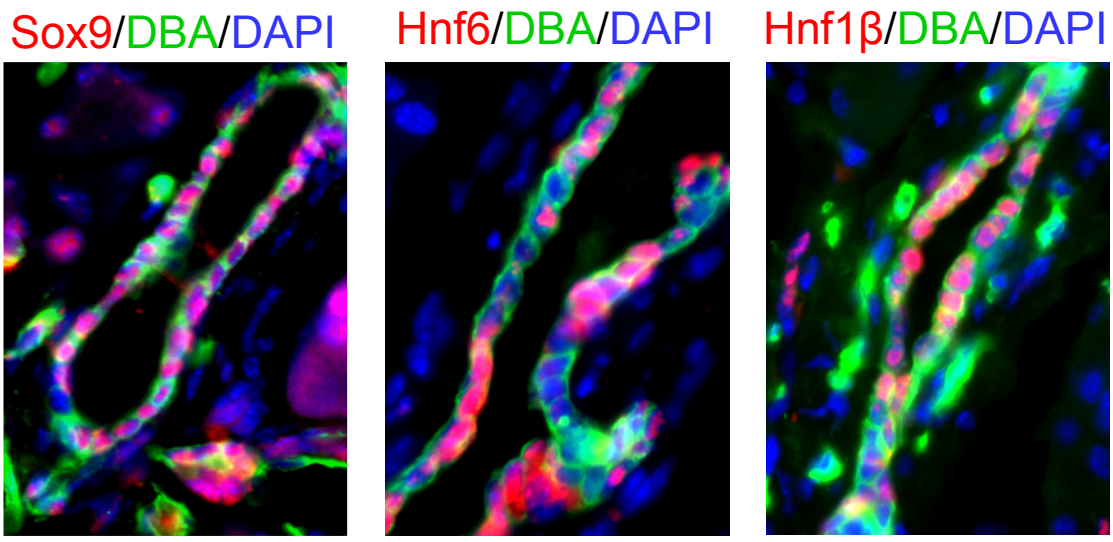


Figure 1.1—Transcription factor expression in adult ductal epithelium. Immunofluorescent labeling for Sox9, Hnf6 and Hnf1β in adult mouse pancreas. Note heterogeneous expression, with neighboring cells often displaying different patterns of transcription factor expression.

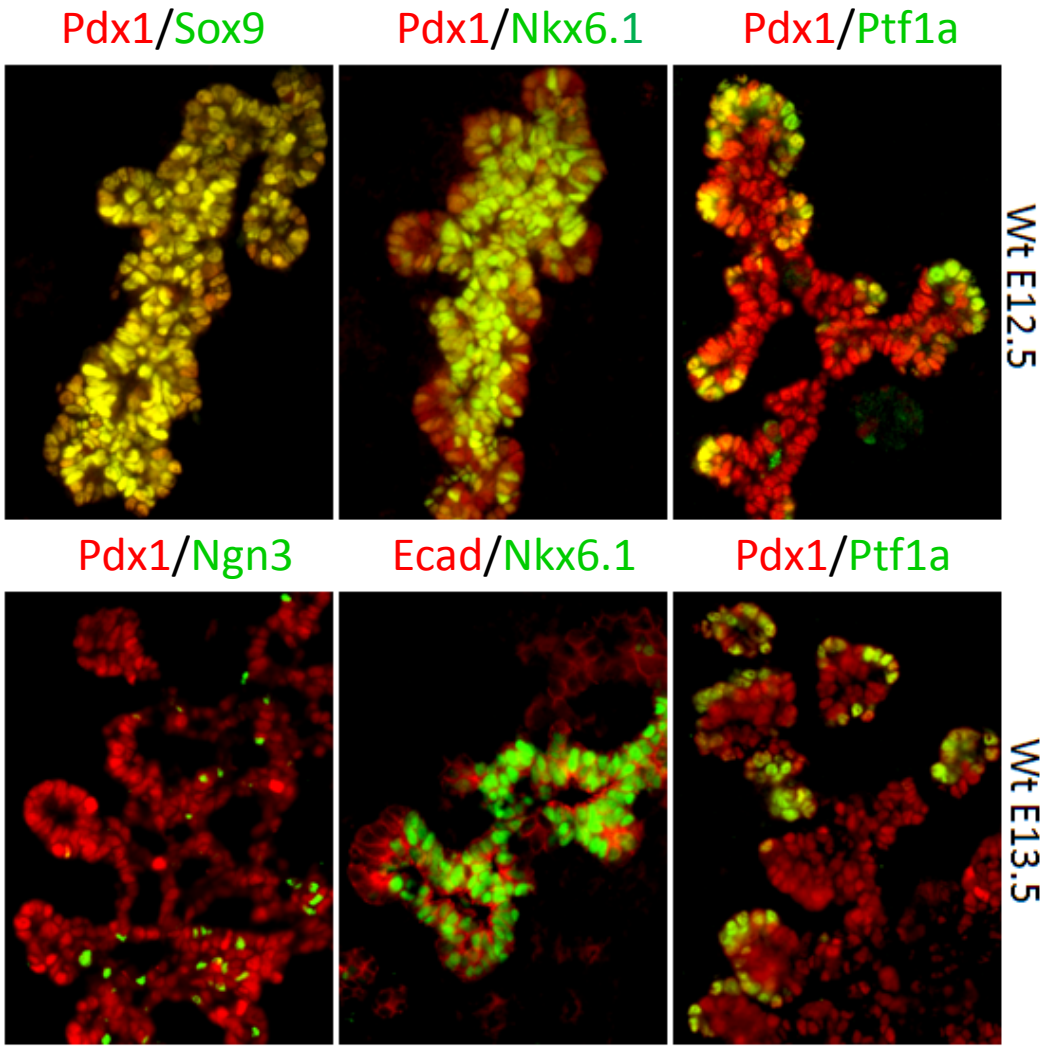
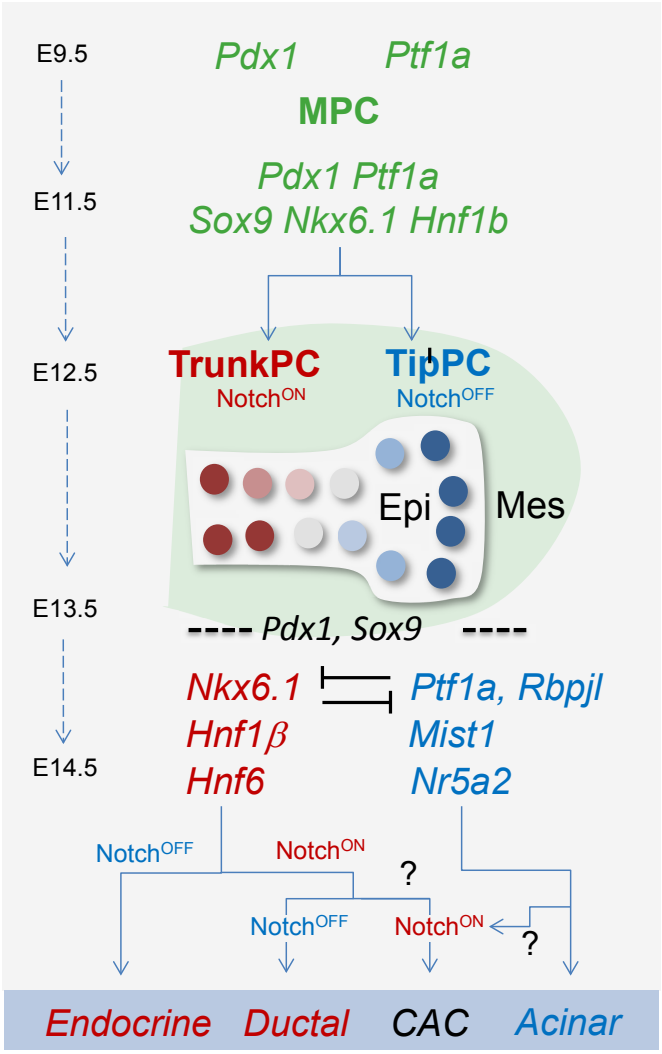


Figure 1.2—Transcription factor topologies in developing mouse pancreas. Immunofluorescent labeling for Pdx1, Sox9, Nkx6.1, Ptf1a, Ngn3 and E-cadherin (Ecad) in emerging trunk and tip domains of developing mouse pancreas. Hnf1β and Nkx6.1 become progressively restricted to the central trunk region of the branching pancreatic epithelium, from which Ngn+ cells begin to emerge. In contrast, Ptf1a is restricted to peripheral tips. As late as E13.5, Pdx1 and Sox9 are expressed in both central trunk and and peripheral tip domains.

Figure 1.3—Emergence of ductal, islet and acinar lineages from progressively restricted trunk and tip progenitor cells. Initially tri-potent multi-lineage progenitor cells (MPCs) expressing both Pdx1 and Ptf1a ultimately give rise to ductal, islet and acinar cells. On E11.5 most MPCs also co-express Sox9, Hnf1β, Hnf6, Nkx6.1, and Nkx6.2. By E12.5, Hnf1β, Hnf6, Nkx6.1, and Nkx6.2 become largely restricted to trunk progenitor cells (TrunkPCs), while Ptf1a becomes restricted to tip progenitors (TipPCs), with Notch signaling promoting the TrunkPC identity. By E13.5, acinar and lslet differentiation have been initiated, associated with the activation of additional lineage-specific transcription factors. Among TrunkPCs, Notch activation promotes a ductal fate. By the time of gestation, active Notch signaling is largely confined to centroacinar cells (CAC), whose derivation from Trunk vs. TipPCs remains unknown. CACs may be derived from either trunk progenitors maintaining active Notch signaling or from tip progenitors reactivating Notch.


E9.5
E11.5
E12.5
E13.5
E14.5
Pdx1
Ptf1a
MPC
Pdx1 Ptf1a
Sox9 Nkx6.1 Hnf1b
TrunkPC
Notch^ON
TipPC
Notch^OFF
Epi
Mes
---- Pdx1, Sox9 ----
Nkx6.1
Hnf1β
Hnf6
Ptf1a, Rbpjl
Mist1
Nr5a2
Notch^OFF
Notch^ON
?
Notch^OFF
Notch^ON
?
Endocrine
Ductal
CAC
Acinar

Chapter 2 – Pancreatic Progenitor Cells in Mice

Introduction

Pdx1 and Ptf1a are expressed very early in pancreatic development. At E11.5, both Pdx1 and Ptf1a are expressed throughout the entire pancreatic epithelium (Figure 2.1), and all epithelial cells at this stage are thought to be capable of forming all types of pancreatic epithelial cells (acinar, ductal and endocrine). By E13.5, Ptf1a is restricted to the "tips" of the pancreas, while Pdx1 is expressed at high levels predominantly in the "trunk" of the pancreas (Figure 2.2); the cells in the tips are fated to become acinar cells while the Pdx1-expressing trunk cells are fated to become endocrine and ductal cells [10].

While the target genes for Ptf1a and Pdx1 are well known in the mature pancreas, the transcriptional targets that regulate early development are not fully known. The goal of this project was therefore to examine the changes in gene expression that take place as the multipotent epithelium develops into the restricted tip and trunk domains. I isolated early progenitors at E11.5, as well as tip and trunk progenitors at E13.5, and performed transcriptional profiling of these cell types. I validated the microarray results using in situ hybridization and available GenePaint in situ data. Finally, I began to characterize the functions of some of these targets using lentiviral shRNA knockdown.

Methods

In order to separate the "tip" progenitor cells from the "trunk" progenitor cells, I needed a way to follow the expression of both Pdx1 and Ptf1a throughout development. To follow the expression of Pdx1, I utilized the well-characterized Pdx1:GFP mouse line developed by Andrew Holland *et. al.* [119]. This is a transgenic mouse line that expresses GFP everywhere Pdx1 is normally expressed. At E11.5, Pdx1 expression can be seen in both

the dorsal and ventral buds, as well as the distal stomach and small intestine (Figure 2.1). By E13.5, when the pancreas has separated into tip and trunk domains, GFP expression is slightly higher in the trunk domains (Figure 2.2). GFP expression becomes progressively more restricted during development and by the time the mouse is an adult, GFP expression is restricted to the islet cells.

Creation of Ptf1a:tdT mice

In order to isolate Ptf1a-expressing cells, we created a knock-in mouse that substitutes the tdTomato fluorophore and an N-terminal Strep-Tag II/Flag-tagged version of Ptf1a (NSF-Ptf1a) into exon 1 of the endogenous Ptf1a locus. The tdTomato fluorophore and NSF-Ptf1a were separated by a sequence coding for the 2A peptide [120], which has a function similar to an Internal Ribosome Entry Site (IRES), although by a different mechanism. The 2A peptide allows both proteins to be transcribed and translated together, but with a failure of peptide bond formation during translation, resulting in expression of the separate proteins at a nearly one-to-one ratio [120]. Since there are no commercially-available ChIP-grade antibodies against Ptf1a, we added the Strep and Flag tags to allow for Ptf1a chromatin immunoprecipitation (ChIP), as well as detection by immunofluorescent labeling (IF) and Western Blot.

To create this mouse, we used ES cells containing the Ptf1a Loxed Cassette Acceptor ($Ptf1a^{LCA}$) allele created by the Magnuson Lab [90]. This allele contains a LoxP site and an inverted LoxP (ILP) site inside the Ptf1a locus. These sites allow for recombinase-mediated cassette exchange, which allows one construct to be easily replaced with another. Our exchange vector replaced exon 1 of Ptf1a with tdTomato, in frame with a sequence coding for the viral 2A peptide, followed by the NSF-Ptf1a

sequence. A TAA stop codon was inserted after the tagged Ptf1a cDNA to prevent expression of the exon 2 (Figure 2.3).

Our genotyping protocol for these mice was based on the absence or presence of the LoxP site. Mice homozygous for the knock-in allele give a 670 base pair band, while mice homozygous for the wild-type allele give a 636 base pair band. Heterozygous mice give both bands. I used the primers F-p48 (5′-CCTTCTGACTTCTCCAAGAAGGCA-3′) and R-p48 (5′-CCCTTTATGCCTGGCATTTCACTG-3′). PCR conditions were: 94°C for 6 min; 40 cycles of 94°C for 1 min., 60°C for 30 s, 72°C for 1 min; 72°C for 7 min. PCR products were run on a 2% agarose gel (Figure 2.4).

Detection of Strep II and Flag Tags

As a positive control for the immunoprecipitation and detection of the NSF-Ptf1a protein using its Flag tag, I transiently transfected HEK293 cells with a PCDNA3.1 vector driving expression of the fusion protein. Cells in 6-well plates were transfected with Lipofectamine 2000 (Life Technologies) following the manufacturer's instructions, and expression allowed to proceed for 36 hr. Cells were then lysed by adding 0.2 mL RIPA buffer to each well (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS) supplemented with Benzonase nuclease (Novagen) and Complete protease inhibitor cocktail (Roche) at the recommended concentrations. Lysis was allowed to proceed for 15 minutes at room temperature with rocking, and samples taken for Western analysis. Lysates were then transferred to 1.5 mL tubes, followed by the addition of 2 µL of Flag-M2 antibody (Sigma). The lysate was added to 20 µL of packed Protein G Sepharose 4 Fast Flow (GE Healthcare) and rotated for 4 hr at 4°C. Resin was then washed 3 times with 1 mL of RIPA buffer and eluted with an equal

volume of SDS-PAGE loading buffer containing fresh reducing agent. In order to retain tdTomato fluorescence for on-blot detection, protein samples in SDS-PAGE loading buffer were not heated.

For Western detection, immunoprecipitated proteins in the eluate were separated by SDS-PAGE and transferred to PVDF membranes using an iBlot device (Invitrogen). The SNAP i.d. device (Millipore) was used according to the manufacturer's instructions to process membranes. Blocking was performed with 0.5% non-fat dried milk in PBST. Flag-M2 monoclonal was used as the primary antibody at a dilution of 1:1000, and goat anti-mouse IgG_1-HRP as the secondary at 1:3000. HRP detection was performed using the Pierce ECL2 kit, followed by chemifluorescent imaging using a Typhoon imager with the recommended settings. To detect tdTomato bands, a second scan was performed with Cy3 filter settings, and the two scan images were superimposed for analysis.

The immunoprecipitation and detection of NSF-Ptf1a from mouse pancreata was performed similarly. Mouse pancreata were homogenized in RIPA buffer at a similar buffer volume to cell number ratio as with the HEK293 positive control cells. Immunoprecipitation and detection protocols were thereafter identical.

Obtaining E11.5 and E13.5 pancreatic epithelium

I crossed the two mouses lines, Pdx1:GFP and Ptf1a:tdT, together to see if both the fluorophores were expressed at the appropriate developmental timepoints. I also wanted to know if I would be able to visualize and sort tip and trunk domains (Figure 2.5).

Acini, ducts and islets all derive from the embryonic epithelium, so it was necessary to separate the epithelium from the mesenchyme. To obtain E11.5 pancreatic tissue, I microdissected the E11.5 epithelium away from the mesenchyme and extracted

the RNA. By E13.5, the mesenchyme is too invaginated in the epithelial folds to be manually dissected, requiring enzymatic digestion and FACS-based cell sorting. I based the enzymatic digestion, FACS and RNA collection method on a previously published method [121]. I crossed Pdx1:GFP $^{Tg/WT}$ mice to Ptf1a:tdT $^{Tg/Tg}$ mice as well as CD1 (Wild-type) mice. This resulted in 4 different genotypes of offspring: GFP $^{Tg/WT}$ /Ptf1a:tdT $^{Tg/WT}$ mice (green and red); GFP $^{WT/WT}$ Ptf1a:tdT $^{Tg/WT}$ mice (red only); GFP $^{Tg/WT}$ /Ptf1a:tdT $^{WT/WT}$ (green only); GFP $^{WT/WT}$ /Ptf1a:tdT $^{WT/WT}$ (no fluorescence). Embryos were screened under a fluorescent microscope during dissection to see which fluorophores they expressed and were separated appropriately. For each FACS day, I dissected 6 to 8 litters with the experimental Pdx1:GFPTg/WT Ptf1a:tdTTg/WT genotype. This yielded, on average, 25 embryonic pancreata. I also dissected an additional 1 to 2 litters to obtain red-only and non-fluorescent controls, which were necessary to set the FACS gates (Figure 2.6)

Enzymatic digestion

Dissected pancreata were placed in scintillation vials with 500 μL of pre-warmed 0.25% Trypsin with EDTA and one small stir-bar per vial. The vials were put on a stir plate and kept at 37 C in the incubator for 10 minutes, which was determined to be the optimal digestion time for our sample (Figure 2.7). After 10 minutes, the reaction was quickly stopped by adding 10% FBS/RPMI-1640. The digested mixture was then filtered through a 40 uM FACS filter cap tube and placed on ice. Samples were immediately sorted with a BD Aria II into a collection tube with 750 μL Trizol LS. On average, I obtained 86,000 Pdx:GFP+ “trunk” cells and 35,000 Pdx:GFP+ Ptf1a:tdT “tip” cells per sorting day.

RNA Extraction

After sorting up to 250 ul of sampleinto Trizol LS, 0.2 ml of chloroform was added for every 750 µL of Trizol LS used. Then sample tube was then shaken vigorously for 15 seconds and left at room temperature for 3 minutes. Samples were then centrifuged for 5 minutes at 12,000 g at 4 C. The aqueous phase of the sample was then transferred to a new tube and 1 volume of 70% ethanol was added. The sample was then pipetted onto an RNeasy mini column in 700 ul aliquots. This column was centrifuged for 15 seconds and the flowthrough was discarded; the column was washed with 700 µL Buffer RW1 and centrifuged again for 15 seconds. The column was then washed twice with Buffer RPE, centrifuging for 15 seconds and 2 minutes, respectively. RNA was eluted with 22 µL of RNase free water and then stored at -80 °C.

Microarray Hybridization

I submitted the purified RNA to the Johns Hopkins Microarray Facility for RIN quality score analysis and subsequent hybridization onto GeneChip Mouse Gene 1.0 ST Array (exon array). The GeneChip 1.0 ST array is a microarray that has, on average, four probes per exon, with an average of over 40 probes per gene. This array can be used with a very small amount of RNA if amplification techniques are used, which was ideal for our experiment as RNA was very limiting. I sent two biological replicates of each of the following: E11.5 epithelium, E13.5 double positive ("tip" cells), E13.5 GFP alone ("trunk" cells). All samples were amplified by the Johns Hopkins Microarray Core with the Nugen amplification kit. I used Partek Genomics Suite and TIBCO Spotfire software as well as assistance from the microarray core to analyze our results.

Validation of Microarray results

I used in situ hybridization instead of qRT-PCR for validation because of limited RNA. Our in situ hybridization protocol was based on a paper by Asp *et al* [122]. To perform the ISH, I designed primers to the gene of interest and amplified the fragment from E13.5 mouse pancreas cDNA. The fragment was run on a 1% agarose gel, stained with GelStar and extracted using a gel extraction kit (Qiagen, Catalog #28704). The purified PCR product was then ligated into the pCR II-TOPO dual promoter vector (Life Technologies, K4600-01) and verified by sequencing. The template containing the in situ probe was amplified from the appropriate plasmid by PCR using the M13F and M13R primers. This PCR product was gel purified and extracted. Next, the DIG-labelled RNA probe was generated in a 20 μL reaction volume: 13 μL of PCR product, 1 μL of RNase inhibitor, 2 μL DIG-labelling mix, 2 μL RNA polymerase T7 or SP6, 2 μL transcription buffer. This reaction was incubated at 37 C for 2 hours, then treated with 2 μL RNase free DNase I and incubated for an additional 15 minutes to digest the DNA. The reaction was then purified on a Qiagen RNeasy spin column, following standard kit instructions and eluted in a volume of 100 μL RNase-free water. 2 μL of RNase inhibitor was added to the eluted RNA probe to increase stability. 2 μL of purified probe was run on a 1% agarose gel to check the quality. This probe was stored at -80 C until needed.

Initially, all ISH was done on E13.5 mouse pancreatic tissue. Tissue was prepared as follows: the dissected pancreata were fixed in 4% PFA at 4 C for 4 hours, then placed in 30% sucrose solution at 4 C overnight. The pancreata were then embedded in OCT and stored at -80 C until cut. Tissue was cut into 20 μm sections and collected on charged slides, which were dried for 5 minutes at room temperature. The tissue on the slides was

then post-fixed in 4% PFA for 10 minutes at room temperature. Slides were washed twice with PBS and then equilibrated for 15 minutes in 5X saline sodium citrate (SSC). Slides were then moved to the prehybridization solution for 2 hours at 58 C in a slide mailer (Ted Pella, catalog #21096). Prehybridization solution consists of 50% deionized formamide, 5× SSC, and 40 μg/ml Salmon sperm DNA. 100 μL of purified probe RNA was added to 6 ml of prehybridization solution, and this mixture was heated to 80°C in order to denature the RNA probe and remove secondary structures. The slides were then incubated with the 6.1 ml of diluted probe in a slide mailer and hybridized for 40 hours at 58-68 C, depending on the probe.

After hybridization, slides were washed twice in 2× SSC for 30 minutes at room temperature, once in 2× SSC at 65°C for 1 hour and once in 0.1× SSC at 65°C for one hour. Next, the slides were equilibrated for 5 minutes in Buffer 1 (100 mM Tris-HCl pH 7.5; 150 mM NaCl). Anti-DIG antibody diluted 1:500 in Buffer 1 with 0.5% blocking reagent (Roche, catalog #11096176001) and slides were incubated in this solution for 2 hours at room temperature. Slides were then washed in Buffer 1, twice for 15 minutes. Next, slides were equilibrated in Buffer 2 (100 mM Tris-HCl pH9.5; 100 mM NaCl, 50 mM MgCl2, 5 mM levamisole) for 5 minutes. For 10 ml of developing solution, 50 μL NBT and 37.5 μL BCIP were added to 10 ml of Buffer 2. Slides were then incubated in this developing solutions for 4 hours to several days, with solution replaced daily. After the color development reaction, the slides were washed in 10 mM Tris-HCl pH7.5, 1 mM EDTA pH 8.0 for 10 minutes. Slides were then washed in 95% ethanol for a few hours to remove background and increase the blue color of the signal. Afterwards, slides were

washed in deionized water for 15 minutes to remove precipitates. Slides were then dehydrated, mounted and imaged.

Functional Assays

I evaluated candidate genes using the dorsal pancreatic bud explant system, which is well-established in the Leach lab [123]. Dorsal pancreatic buds are isolated from E11.5 mouse embryos and grown in cell culture medium containing 10% Fetal Bovine Serum, 1% Anti-Anti in Medium 199. They will grow and differentiate for about 1 week. Various factors (siRNA, lentiviral vectors, morpholinos, etc.) can be added to the medium and the effects of these factors can be observed in real time. I separated the epithelium and mesenchyme in order to selectively knock down genes of interest in the epithelium—the origin of the acini, ducts and islets. The epithelium was placed directly into cell culture medium in a 24 or 96 well plate, while the mesenchyme was placed on top of a cell culture filter touching the medium (Figure 2.8) (Millipore, catalog #PICM01250). Lentiviral shRNA was added to the wells containing the epithelium for 24 hours. After 24 hours, the media was changed and epithelium and mesenchyme were recombined. Subsequently, media was changed every other day. After 1 week in culture, I imaged the dorsal buds to visualize the Pdx:GFP/Ptf1a:tdT domains, as compared to control dorsal bud explants.

Results

Validation of Ptf1a:tdT mouse

Pancreata from adult Ptf1a:tdT mice were examined, showing that tdTomato expression was evident in adult exocrine acini, but absent from islets, ducts and blood vessels (Figure 2.9). The pancreas appeared entirely normal by H&E (Figure 2.10), and

mice survived as homozygotes, implying that our affinity-tagged version of Ptf1a remained entirely functional (Figure 2.11).

Immunoprecipitation of NSF-Ptf1a

The tdTomato-NSF-Ptf1a construct used in transgenic mice was expressed in HEK293 cells, and the use of Flag-M2 antibody for immunoprecipitation and Western detection of the fusion protein was evaluated (Figure 2.12). It was found that NSF-Ptf1a could be detected by Western in cell lysates, and could be immunoprecipitated using Flag-M2 antibody and protein G beads. In addition, the co-translationally expressed tdTomato reporter could be detected by intrinsic fluorescence scans of Western blots. Comparison with molecular weight standards indicated that these proteins were correctly cleaved at the 2A peptide into separate proteins of the expected size. I found that the Strep II tag could also be used with Strep-Tactin resin for protein capture, but with less efficiency than Flag immunoprecipitation (data not shown).

Although I was able to detect both the Strep II tag and the Flag tag by Western in HEK293 cells that were transfected with the tdTomato-NSF-Ptf1a construct, I was unable to detect either tag in lysates from transgenic mouse tissue. Attempted immunoprecipitation from mouse tissue also did not enrich the NSF-Ptf1a protein enough to enable detection. I sequenced DNA from the region of interest in the mouse and confirmed that our construct was in the correct location with no mutations. It is likely that the expression levels of Ptf1a from its native locus during mouse development are significantly lower than expression levels resulting from transfection into HEK293 cells. This result may therefore simply be the result of insufficient sensitivity of the Flag western detection and IP when used with proteins expressed at very low levels.

Alternatively, pancreas-specific post-translational processing or protein folding might lead to epitope masking. Therefore, although we did utilize the tdTomato reporter as a marker to isolate cell populations by FACS, we did not attempt to perform Ptf1a ChIP using the Flag tag.

Microarray Results

We compared RNA from E11.5 epithelium, E13.5 trunk cells and E13.5 tip cells using the Affymetrix Exon Array and analyzed the data using the Partek Genomics Suite. With the Partek software, we generated a Principle Component Analysis (PCA) plot, which explains the variation between the samples. PCA component #1 explains the majority of the variation, followed by PCA components #2 and #3. The closer the replicates are to each other, the more reproducible the data is. Our replicates are reasonably clustered together, considering the heterogeneity of mice (Figure 2.13). The Affymetrix gene chip array covers all genes in the mouse genome; I limited my study to genes that were expressed at least 2 standard deviations higher than the mean in either population. For the tip versus trunk comparison, this yielded 763 genes expressed at higher levels in the trunk and 487 expressed at higher levels in the tip (Figure 2.14)

I then limited my analysis to transcription factors, since transcription factors are more likely to have a functional effect on pancreas development. This reduced the number of genes in the trunk from 763 genes to 31 genes and in the tip from 487 genes to 27 genes (Figure 2.15). Our results were filled with many genes already well known in pancreas development, expressed in the appropriate region, which confirmed the validity of our screen. Ptf1a, the gene on which our sorting was based, was shown to be higher in the tip cells, along with one of its binding partners, Rbjl. Other acinar specific genes such

as Nr5a2, along with many digestive enzymes were confirmed to be higher in our sorted tip population. Similarly, our trunk population contained many genes associated with endocrine and duct formation, such as Pax4, Pax6, Neurod1, Neurog3 and Nkx6-1.

In Situ Hybridization Validation of Microarray Results

Transcription factor candidates identified by microarrays were further validated by probing their expression patterns through *in situ* hybridization and, if antibodies were available, by immunofluorescence. I successfully validated the localization of ASCL2, a transcription factor expressed higher in the tips. ASCL2 shows expression in the tip cells at E13.5 through E15.5 by both in situ hybridization and immunofluorescence. After E16.5, ASCL2 can no longer be detected at higher levels in the tips by either in situ hybridization or immunofluorescence (Figure 2.16, Figure 2.17). LHX1, another transcription factor which microarray data indicate is expressed at higher levels in the trunk, could also be validated based on in situ images in GenePaint [124] (Figure 2.18).

Functional Assays in Dorsal Bud Explants

I performed some preliminary lentiviral shRNA knockdown experiments to probe the function of transcription factors with expression patterns verified as tip- or trunk-specific by microarray and ISH. I expected that knockdown of genes expressed in the tip cells would prevent, delay or reduce formation of tip domains; while knockdown of genes expressed in the trunk would prevent, delay or reduce formation of trunk domains.

For ASCL2, a little more than half of the of the ASCL2 shRNA lentiviral infected buds are affected in one of two ways. In phenotype I, the buds have very low levels of Ptf1a:tdT compared to controls (Figure 2.19 and Figure 2.20). In phenotype II, the buds express Ptf1a:tdT but fail to form normal tip structures (Figure 2.19 and Figure 2.20).

Phenotype I has never been seen in controls, while 18% of ASCL2 knockdown buds have this phenotype. Phenotype II was seen in 8% of controls and 36% of ASCL2 knockdown buds.

Dorsal buds treated with LHX1 shRNA tend to show an increase in the number tip structures. Control buds typically have 17-24 "tips" (67%), although some control buds have 25-29 tips (33%). For the LHX1 knockdown buds, 38% has 17-24 tips, another 38% had 25-29 tips, and 25% had greater than 30 tips (Figure 2.21 and Figure 2.22).

Discussion

In this study, we created a transgenic mouse expressing NSF-Ptf1a from its native locus, along with a tdTomato reporter. The pancreata from these mice developed normally even when homozygous for the transgenic allele. Because Ptf1a is absolutely required for the development of the pancreas, this indicated that there was no disruption to the function of Ptf1a. When crossed with the well-characterized Pdx1-GFP mouse, the tdTomato-NSF-Ptf1a mouse enabled double-fluorescent mice to be made, allowing for cells of the "tip" and "trunk" domains of the mouse pancreas to be visualized during development and separated by FACS after enzymatic dissociation.

Given our ability to selectively label tip and trunk cells, the goal of our project was to use microarrays to identify any transcription factors affecting development of these tissues. Once Pdx1 and Ptf1a become localized to tip and trunk domains, it is not feasible to separate these domains by manual dissection. Therefore, enabling the cells of these domains to be separated by FACS was essential to the goals of project, and allowed enough RNA to be obtained from tip and trunk cells for microarray analysis and comparison of differentially-expressed transcription factors.

After comparison of microarray data between tip and trunk cells, differentially-expressed transcription factors were identified, and as many as possible were validated by comparison with the literature and, for novel transcription factors, by in situ hybridization. Two transcription factors were initially identified which had no previously known role in pancreas development, and which could be verified by ISH to be expressed in the expected domain of the pancreas.

ASCL2

Ascl2/Mash2 is a class II basic helix-loop-helix transcription factor that is a member of the mammalian Achaete-Scute family. It binds to the E box sequence 5'-CANNTG-3 and heterodimerizes with other basic helix-loop-helix proteins to enhance its own DNA binding [125]. Ascl2 is a target for Wnt signaling, containing a binding site for TCF-4 in its promoter [126]. It is an imprinted gene located in the gene cluster along with H19, IGF-2, and Ins2. ASCL2$^{-/-}$ mice die by E10.5 from placental failure, and the same is true for mice with only a paternal copy of Ascl2 [127, 128]. Due to this lethality, conditional knockouts must be made in order to study the role of ASCL2 in other tissues. Therefore, Ascl2 has not been extensively characterized with respect to its role in the development.

Nevertheless, a small number of conditional knockouts have been studied. For instance, a mouse with a floxed version of Ascl2 was created by the Clevers lab [129] and crossed to another mouse line with Cre recombinase under the control of villin, an intestinal-specific promoter. This eliminated Ascl2 specifically in intestinal cells, resulting in a loss of stem cells in the small intestine. Conversely, this group also created a transgenic mouse that forced Ascl2 to be expressed throughout the small intestine, resulting in intestinal hyperplasia [129]. In addition, the floxed Ascl2 mouse line was

recently crossed to a CD-4 Cre recombinase line. This resulted in mice with immune defects such as reduced anti-viral IgG production and greatly diminished germinal center B-cell formation [130]

Ascl2 is upregulated in colorectal adenocarcinomas, and shRNA knockdown of Ascl2 in HT-29 cells, a colorectal cancer line, decreased the sphere forming ability of these cells [131, 132]. However, levels of Ascl2 mRNA do not seem to have an effect on the survival of colorectal cancer patients or their chance for reoccurrence of cancer. In addition, ectopic expression of Ascl2 did not increase tumor progression in the APC^{Min} mouse model of colon cancer [133].

LHX1

LHX1, also known as Lim1, is a homeobox gene that is required for extraembryonic tissues and head formation in the mouse [134, 135]. Because LHX1 is so vital to the developing embryo, conditional knockouts are required to examine that role of LHX1 in different tissues. Using a floxed LHX1 mouse strain and various Cre recombinase lines, LHX1 has been shown to be important in duct development in several different tissues, such as the female reproductive system and the urogenital system. In the female reproductive system, LHX1 is required for proper formation and extension of the Mullerian duct. Conditional knockout of LHX1 under control of the Wnt7a promoter resulted in failure of Müllerian duct extension, and consequently uterine aplasia [136]. When LHX1 is conditionally knocked out under the control of the Pax2 promoter, the nephric duct fails to fully extend, and the ureteric bud does not form properly [137]. Thus, given our findings of a role of LHX1 in pancreatic “trunk” progenitors, which go

on to form duct and islet cells, this suggests that LHX1 may have a common role in duct formation in many different tissues.

Significance of Results

We have created an entirely new mouse line which can be used for further pancreas studies or other tissues expressing Ptf1a, such as the retina. I have completed transcriptional profiling of E11.5 early pancreatic progenitors as well as E13.5 tip and trunk progenitors and generated lists of genes that differ significantly between these populations.

Ultimately, conditional knockout mice for Ascl2 and LHX1 will yield more information on the roles of these two genes in pancreas development.

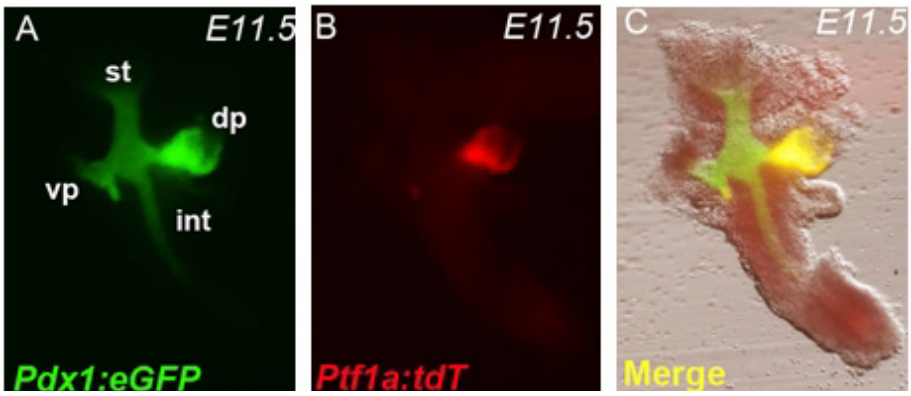


Figure 2.1—Expression of Pdx1 and Ptf1a in the E11.5 pancreas. Both Pdx1 (green) and Ptf1a (red) are expressed throughout the entire pancreatic epithelium.

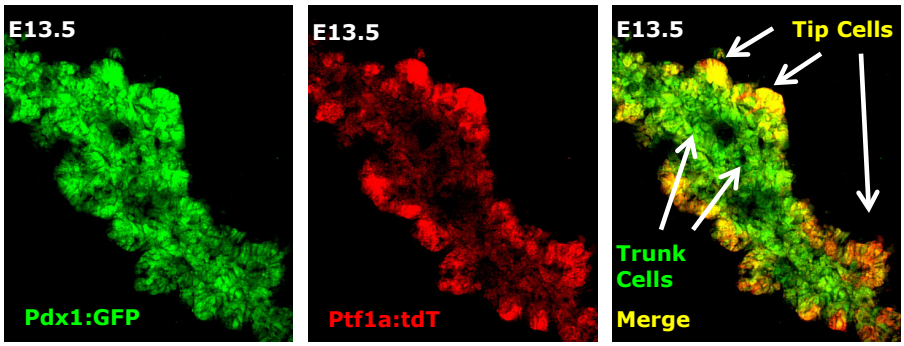


Figure 2.2—Expression of Pdx1 and Ptf1a in the E13.5 pancreas. Pdx1(green) is expressed is both the tip and trunk domains at E13.5 while Ptf1a (red) is now restricted to the tips.

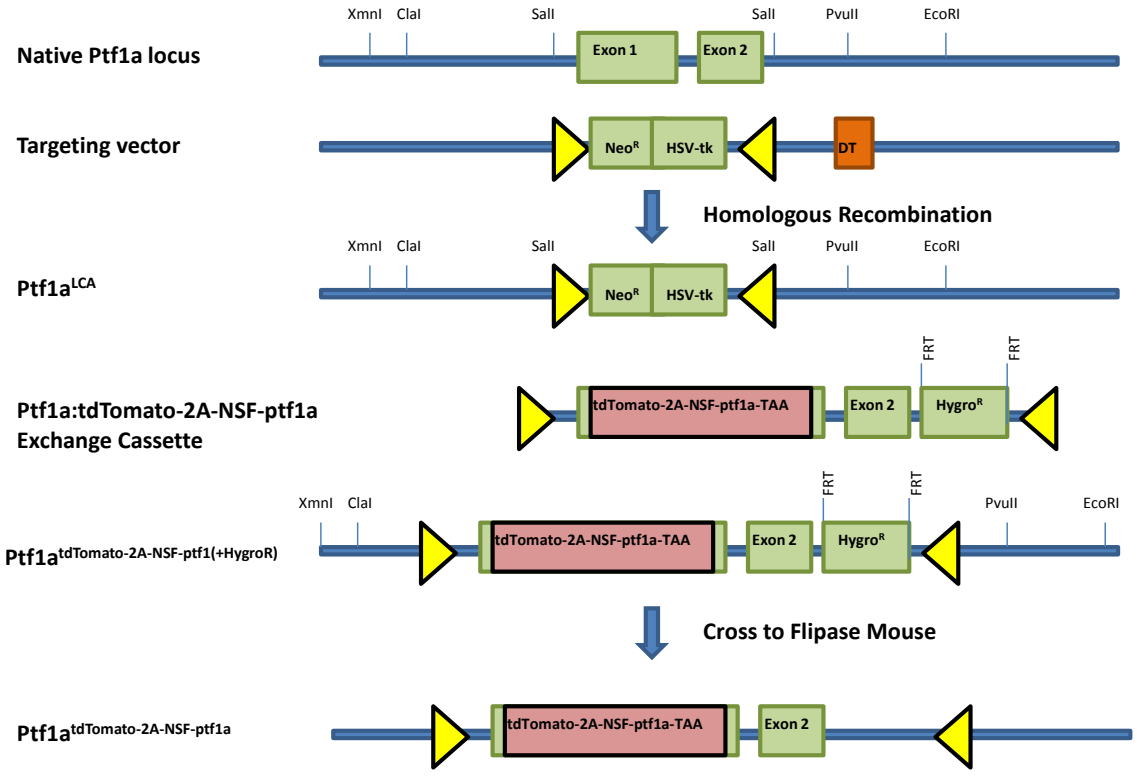


Figure 2.3—Gene targeting strategy for the Ptf1a:tdT mouse. The Ptf1aLCA locus substitutes NeoR-HSV-tk into the endogenous Ptf1a locus inside inverted LoxP sites. For our construct, we replaced all of Ptf1a exon1 with tdTomato-2A-NSF-ptf1a, followed by TAA. The mouse was then crossed to a Flipase mouse to remove the FRT sites.

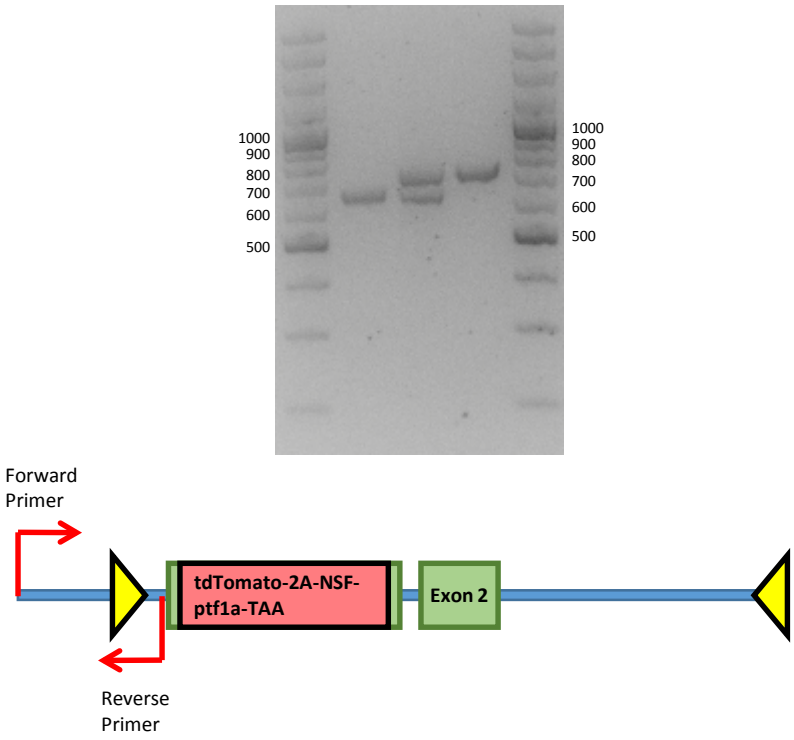


Figure 2.4—Genotyping the Ptf1a mouse. DNA fragments amplified by PCR from wild type, heterozygous and homozygous knock-in mice are shown. The wild-type allele yields a 636 bp band and the Ptf1a knock-in allele yields a 670 bp band (top). Diagram of primer location for Ptf1a:tdT PCR (bottom).

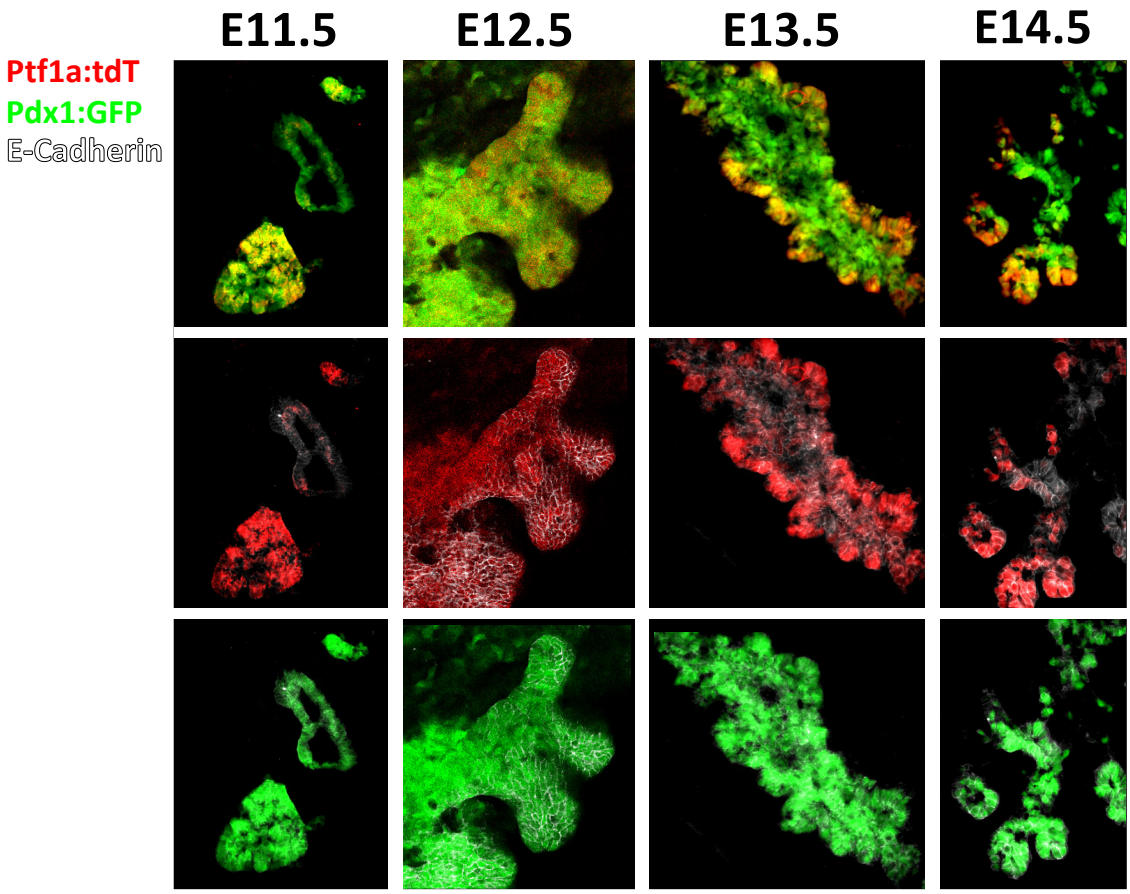


Figure 2.5—Ptf1a and Pdx1 expression vs. embryonic stage. At E11.5, both Ptf1a and Pdx1 are expressed throughout the epithelium. As development progresses, Ptf1a becomes restricted to the tip domains while Pdx1 is expressed at higher levels in the trunk.

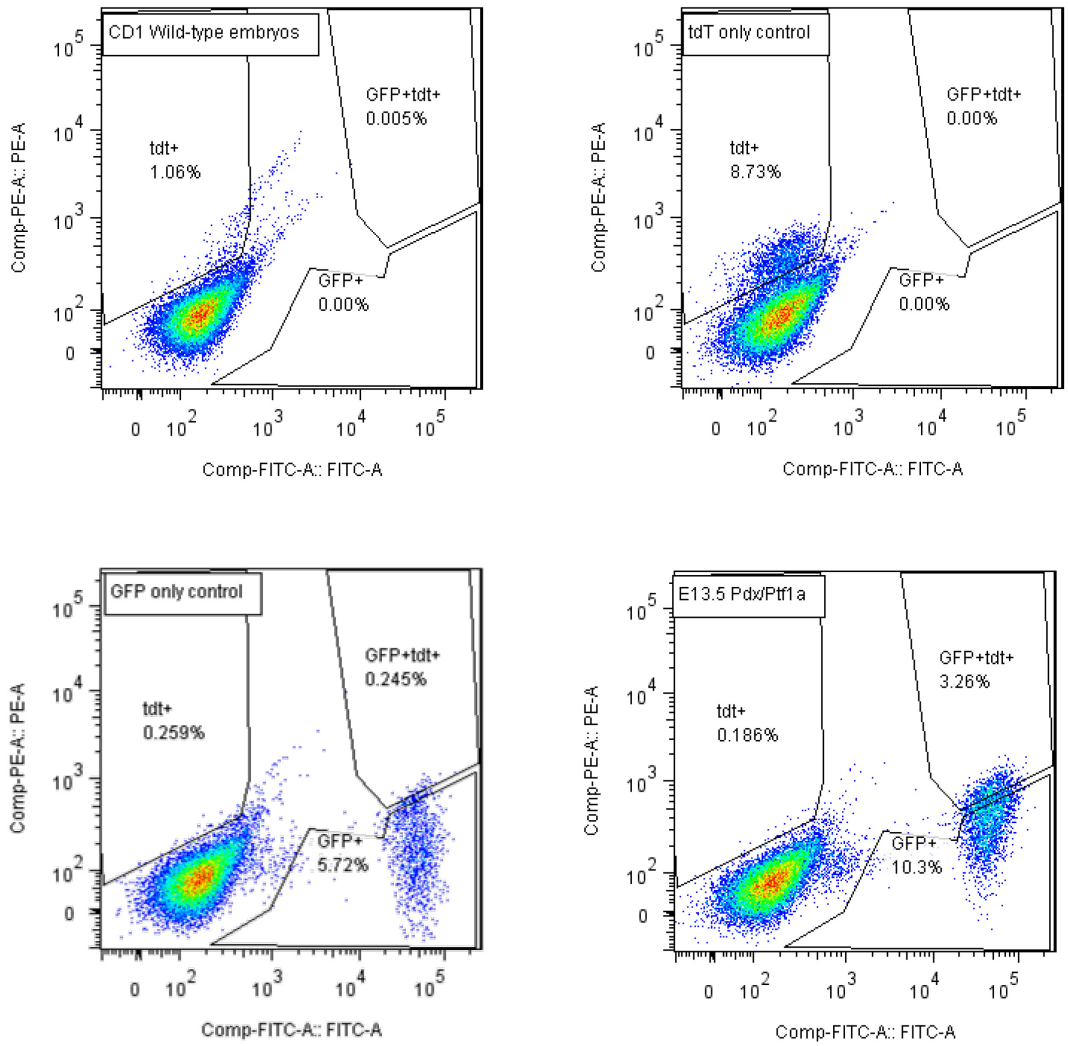


Figure 2.6—FACS gates for RNA isolation. The red-only, green-only and non-fluorescent pancreata were used to set the FACS gates.

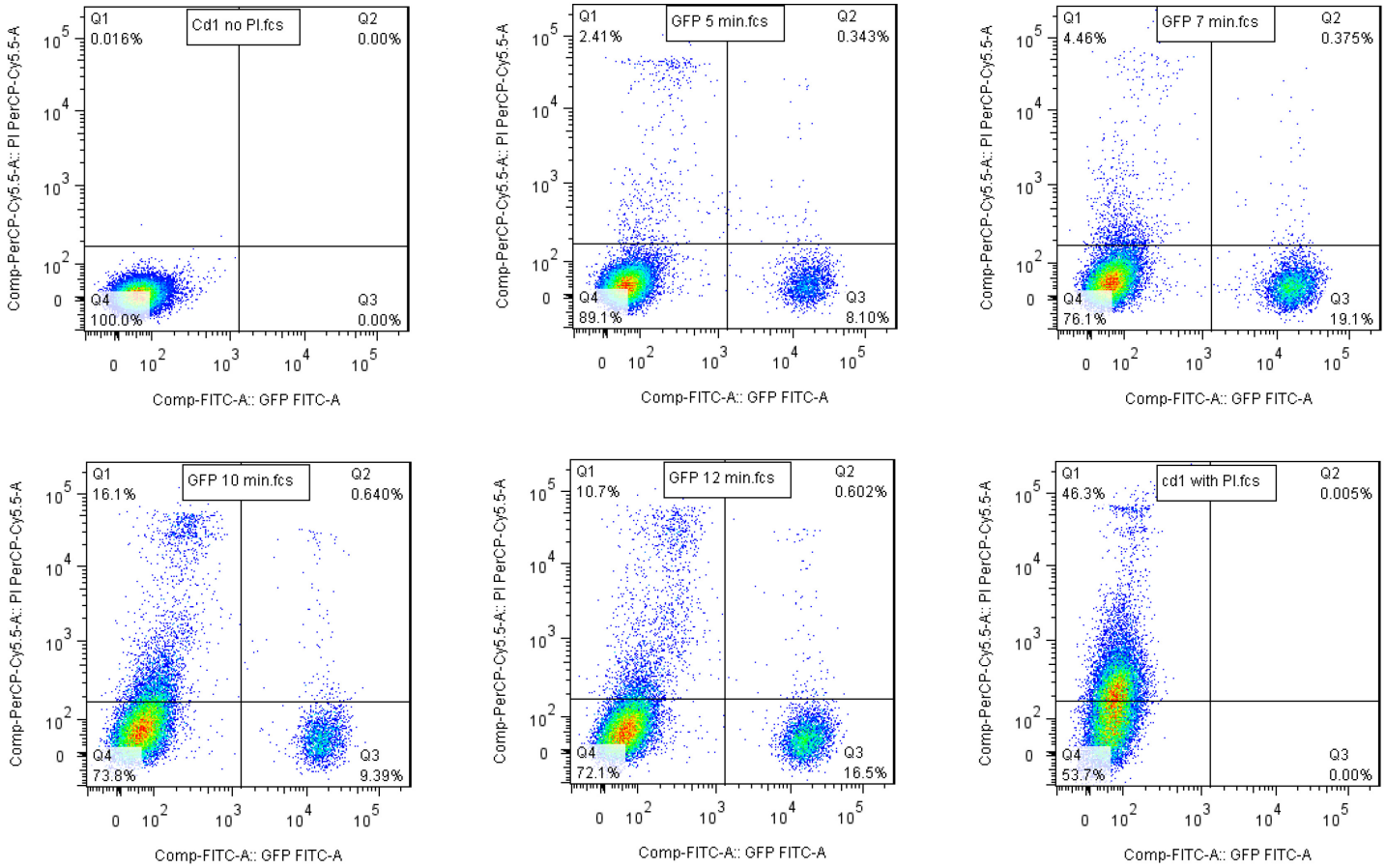


Figure 2.7—Determination of optimal protease digestion time for embryos. I determined the optimal digestion time Pdx:GFPTg/WT embryos and propidium iodide (PI), which stains dead and dying cells. I wanted to maximize the number of Pdx:GFP+ cells liberated while minimizing cell death, indicated by PI staining. 10 minutes was found to be the optimal digestion time.

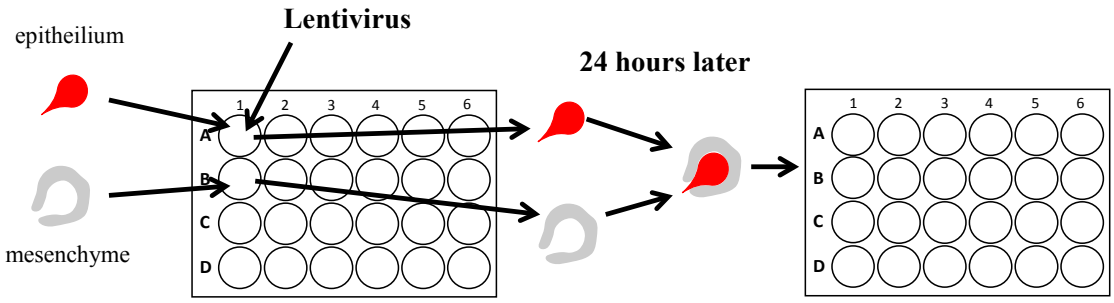


Figure 2.8—Dorsal Bud Culture System. Epithelium and mesenchyme are separated by dissection. The mesenchyme is placed on a cell culture insert filter and the epithelium is incubated with lentiviral shRNA for 24 hours. After 24 hours, the epithelium and mesenchyme are recombined and allowed to grow for an additional 6 days.

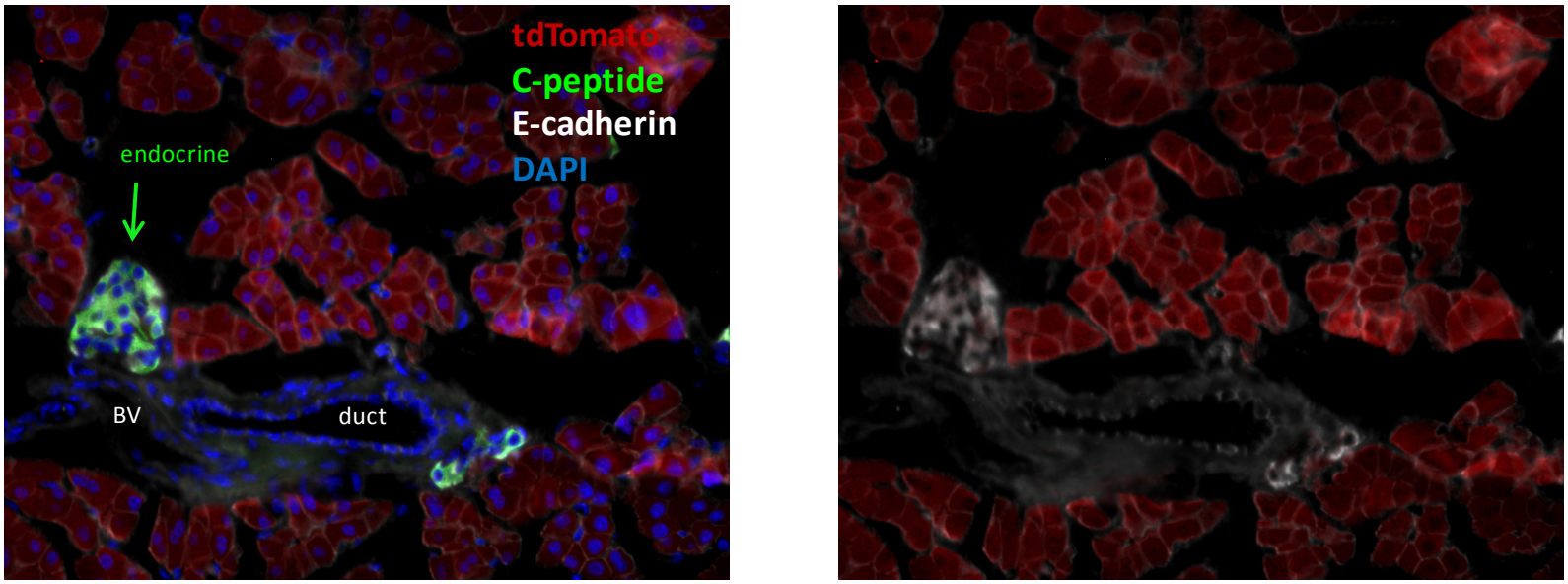


Figure 2.9—Expression of Ptf1A in the adult mouse. tdTomato is expressed in the acini but is absent from ducts, blood vessels (BV) and endocrine cells.

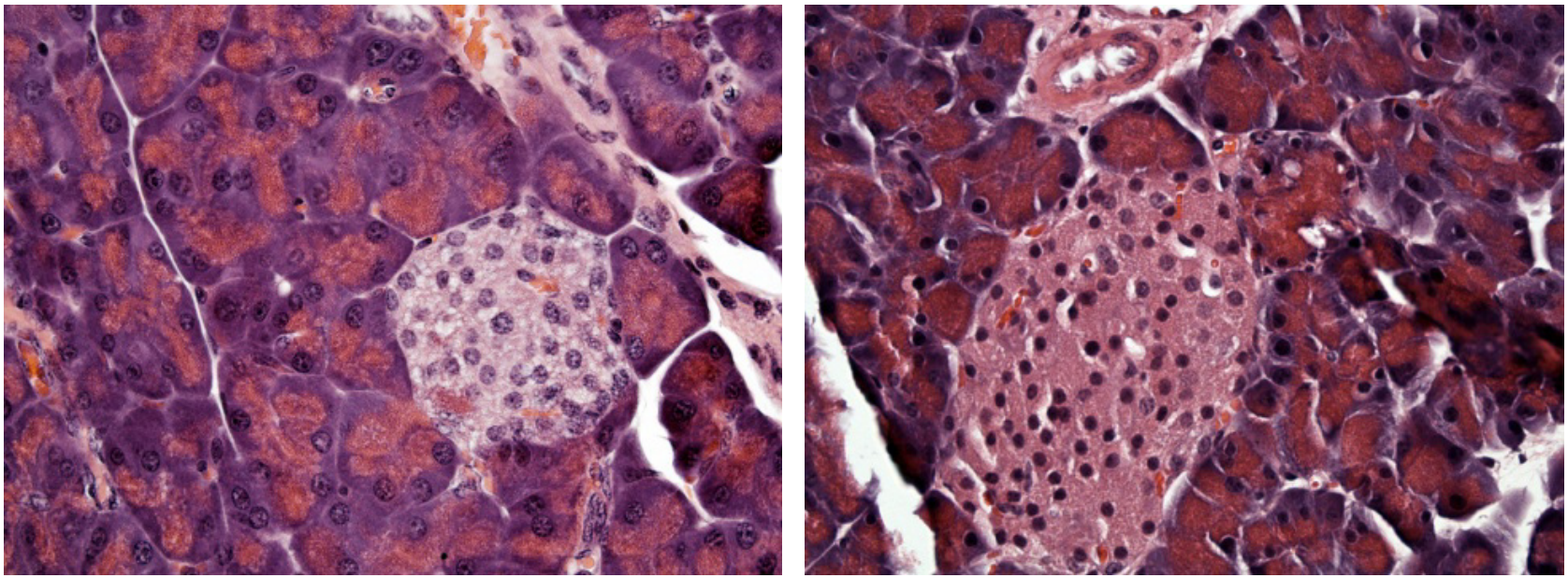


Figure 2.10—H&E staining of pancreas from Ptf1a mice. The pancreas develops normally in Ptf1a:tdTomato heterozygous (left) and homozygous (right) mice.

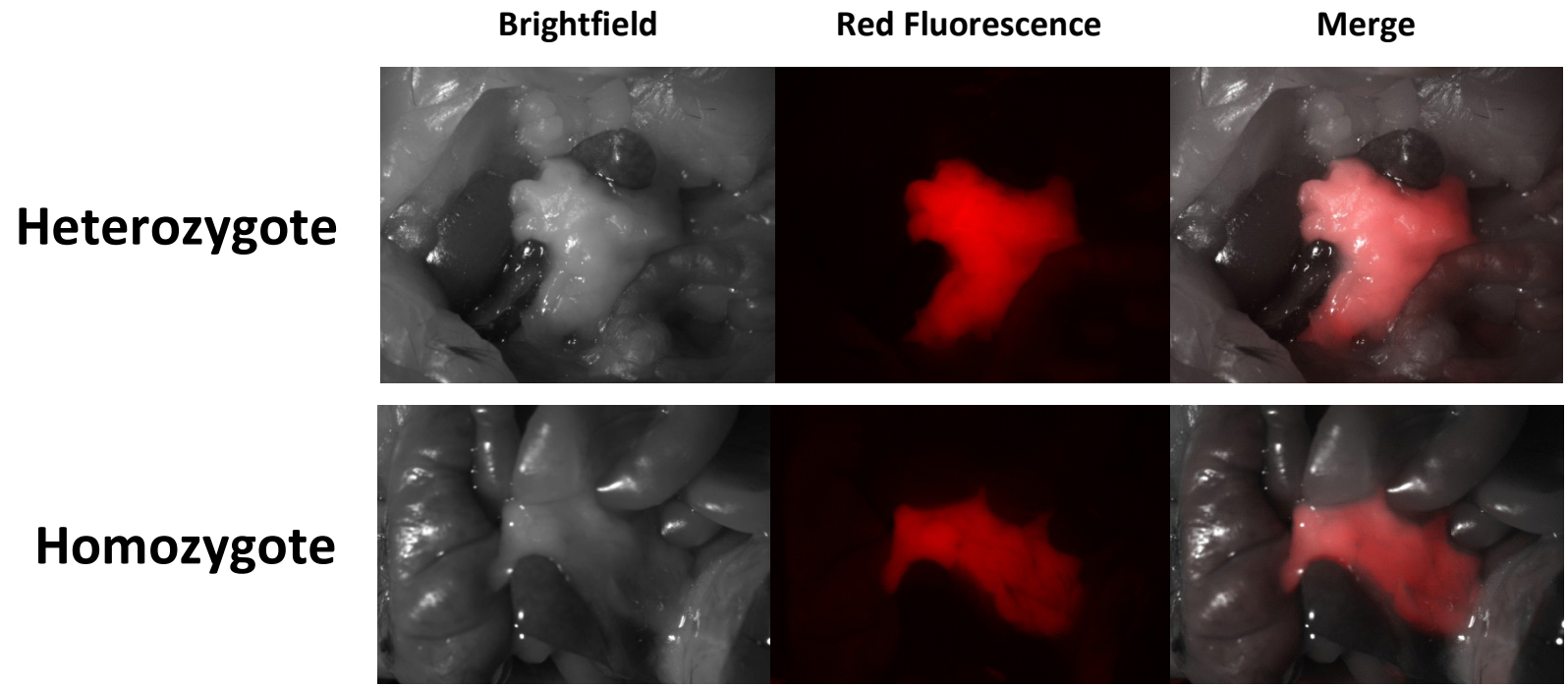


Figure 2.11—Visualization of Ptf1a expression using the tdTomato reporter. Both Heterozygous and Homozygous Ptf1a:tdT mice express tdTomato at approximately the same intensity.

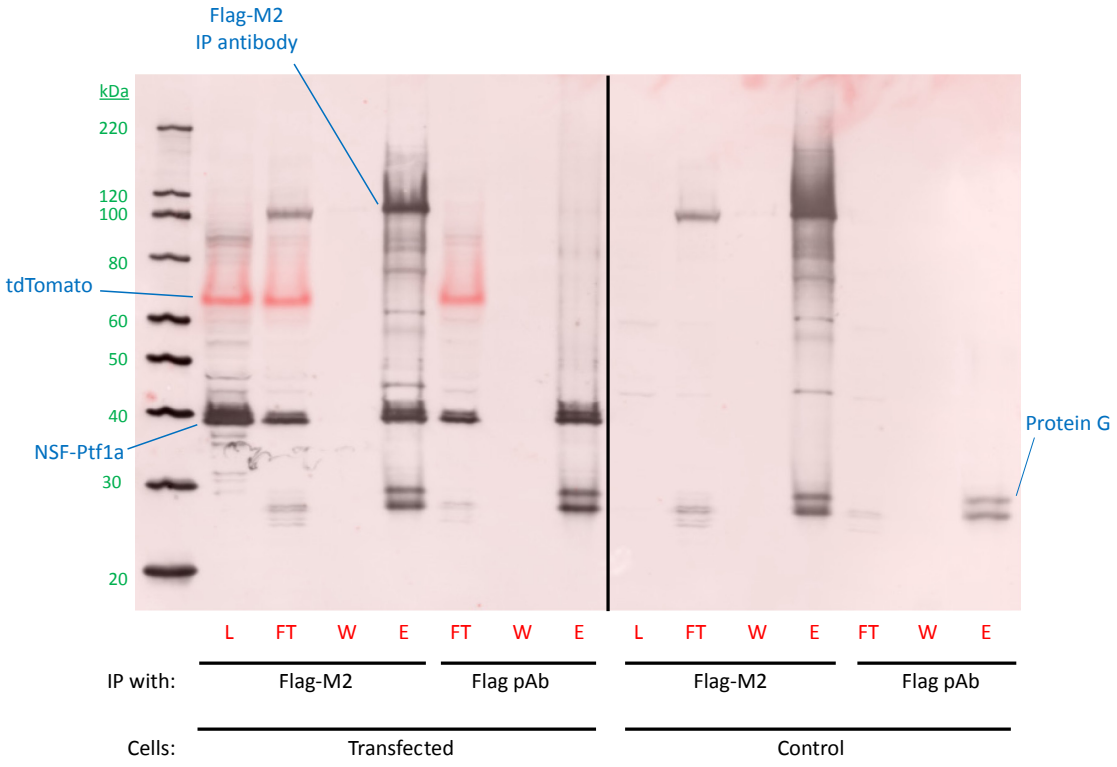


Figure 2.12—Immunoprecipitation of NSF-Ptf1a from HEK293 cells. NSF-Ptf1a is expressed in HEK293 cells, and can be precipitated by the Flag-M2 monoclonal or a polyclonal flag antibody (**L**oad, **F**low**t**hrough, **W**ash and **E**lution from immunoprecipitation are shown). Because an anti-mouse HRP secondary antibody is used for detection, the mouse-derived Flag-M2 IP antibody is also detected. Black bands show antibody staining; red bands show direct detection of tdTomato fluorescence, demonstrating correct separation of the co-translationally expressed tdTomato reporter from NSF-Ptf1a protein.

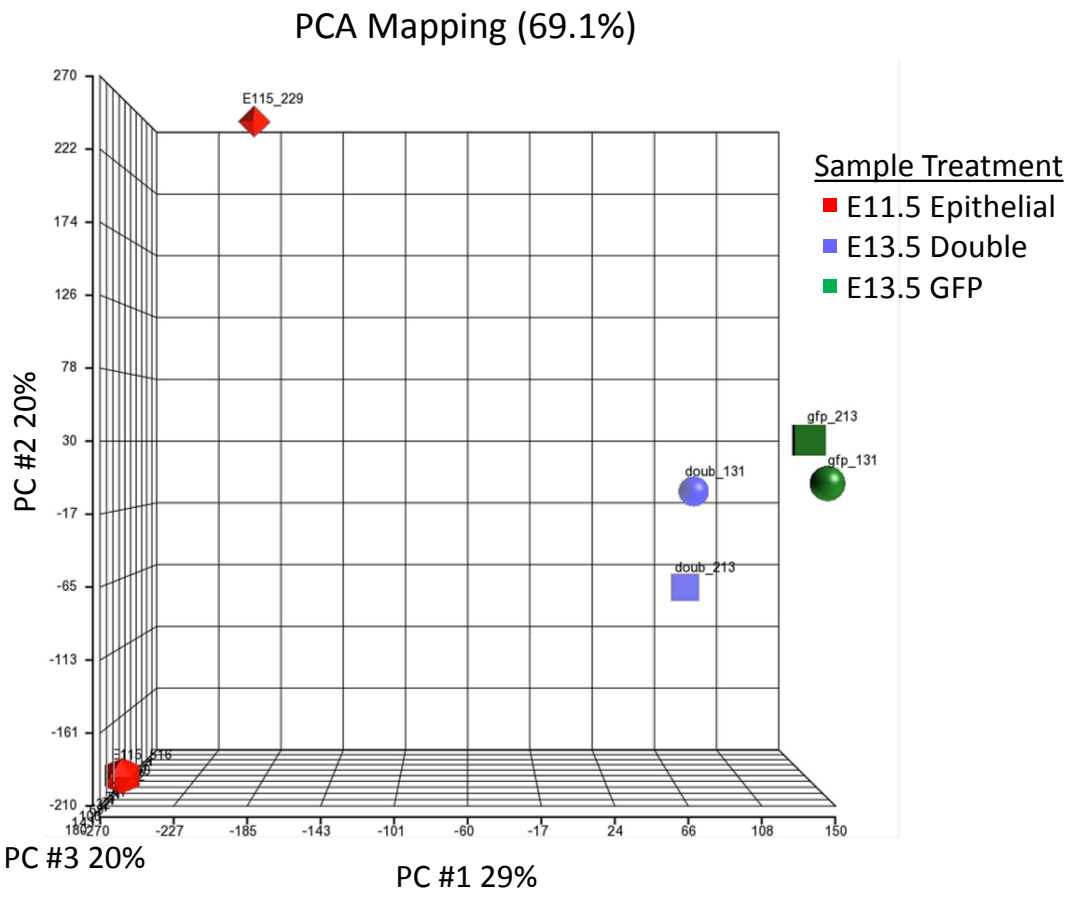


Figure 2.13—Principle Components Analysis. Plot shows the similarities among samples submitted. Principle component #1 (x-axis) explains 29% of the variation, principle component #2 (y-axis) explains 20% of the variation and principle component #3 (z-axis) explains 20% of the variation.

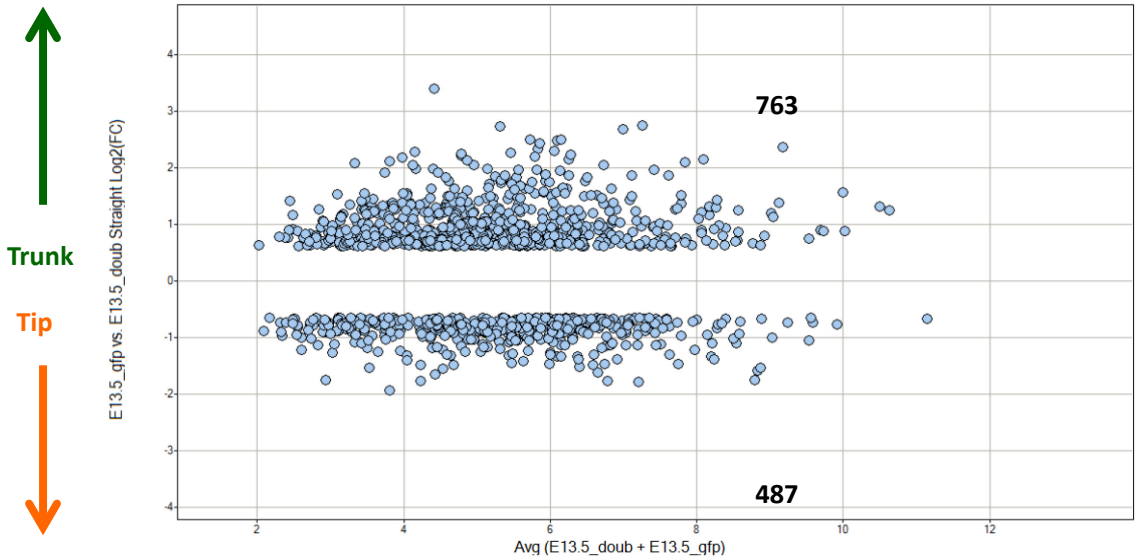


Figure 2.14—Minus versus Average (MvA) plot of all genes with at least 2 standard deviation difference between tip and trunk. The Y axis measures the difference in expression of trunk vs tip and the X axis measures the intensity of the signal compared to the average signal on the GeneChip. A probe's position on the X axis is strongly correlated with GC content; that is, probes with higher GC content are farther on the right.

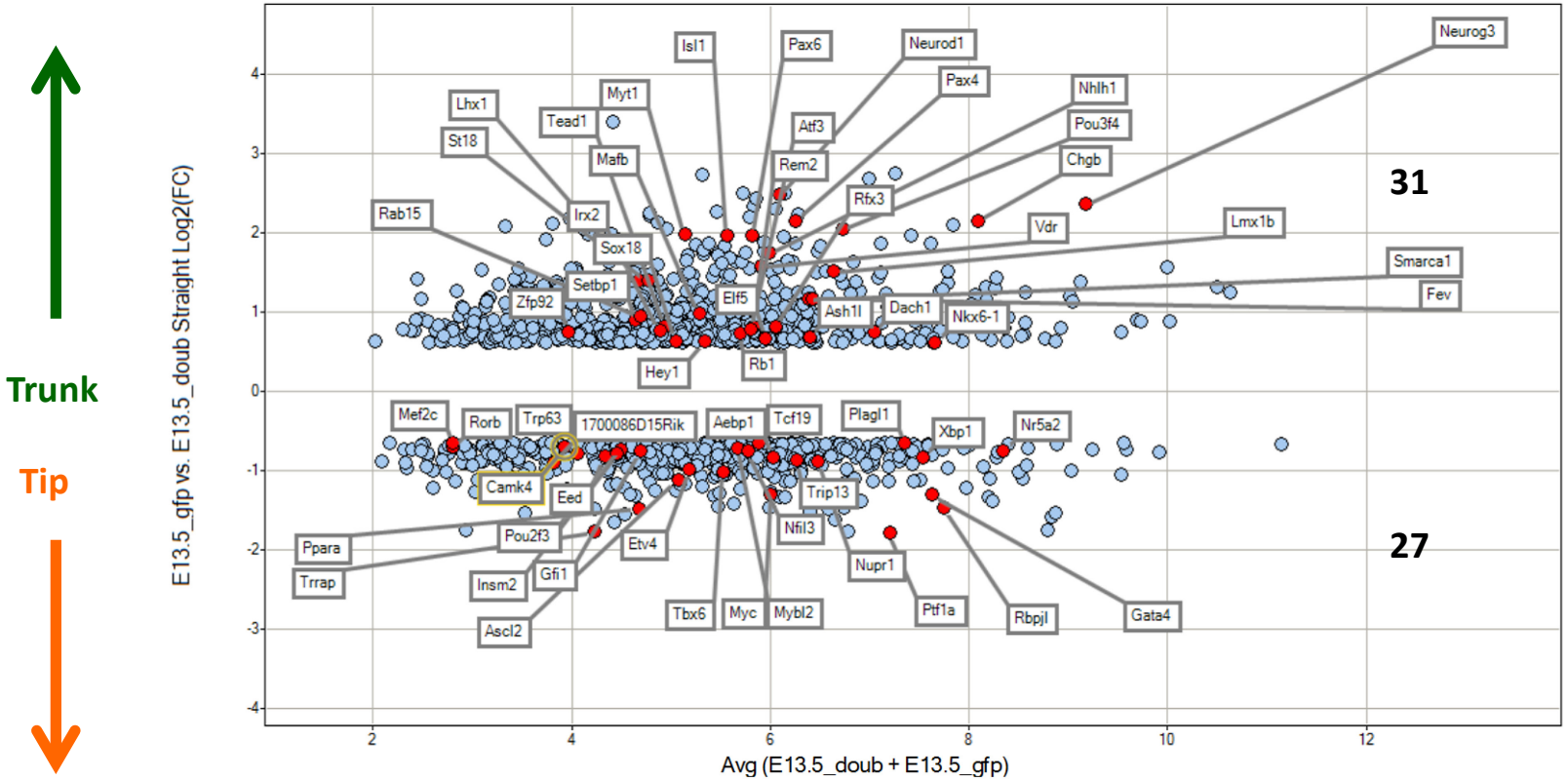


Figure 2.15—MvA plot with transcription factors highlighted.

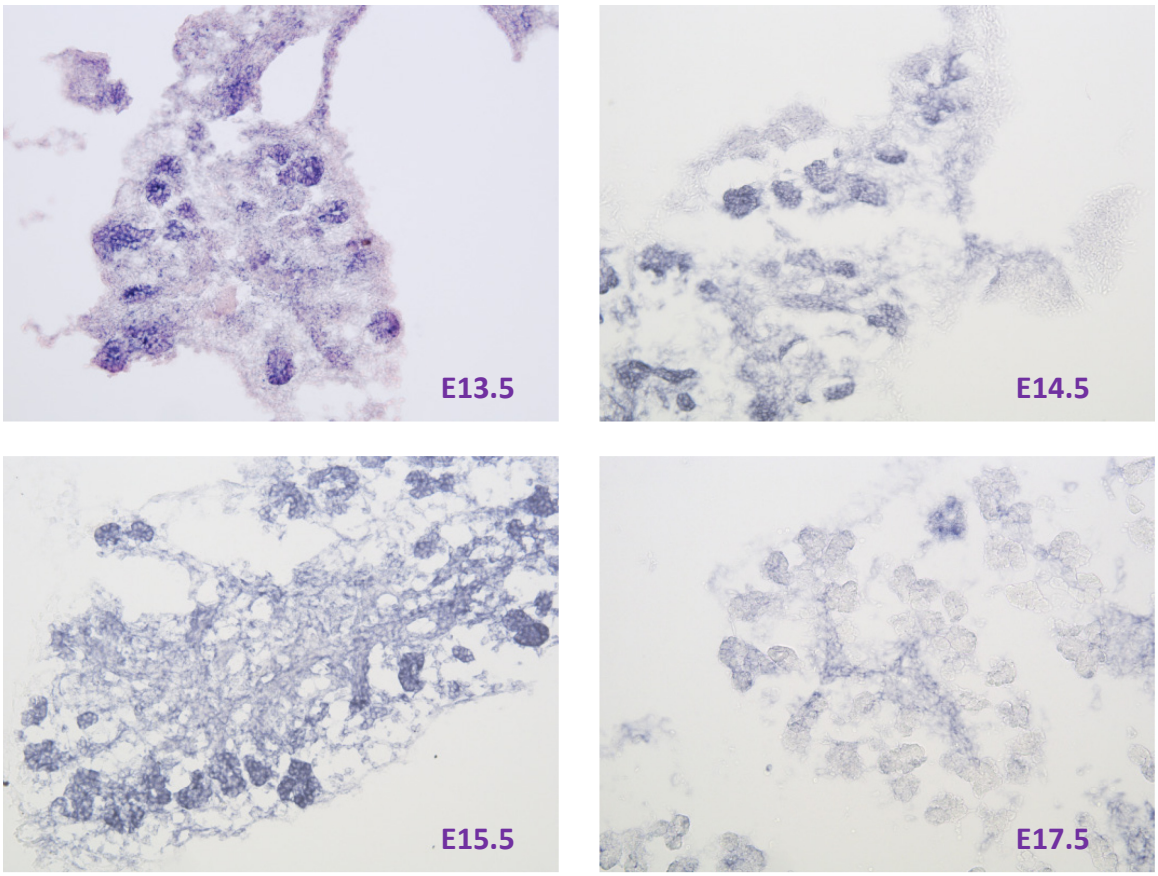


Figure 2.16—Developmental series of ASCL2. ASCL2 is a transcription factor that is expressed in the tip region from E13.5 to E15.5. It does not appear to be expressed in differentiated acinar cells.

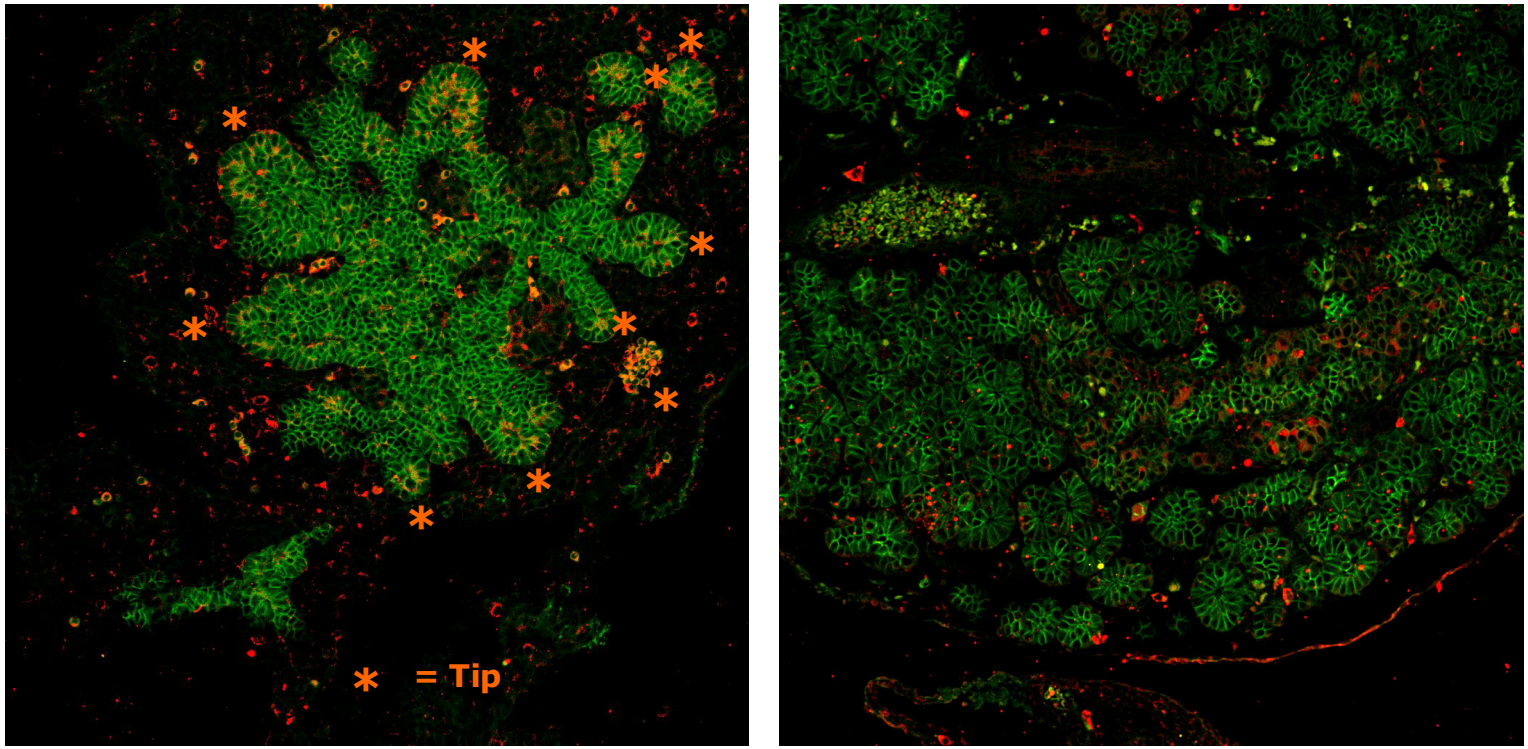


Figure 2.17—Immunofluorescence images of ASCL2 at E13.5 and E16.5. The Ascl2 protein is also localized to the tips at E13.5 and absent from the epithelial tissue by E16.5

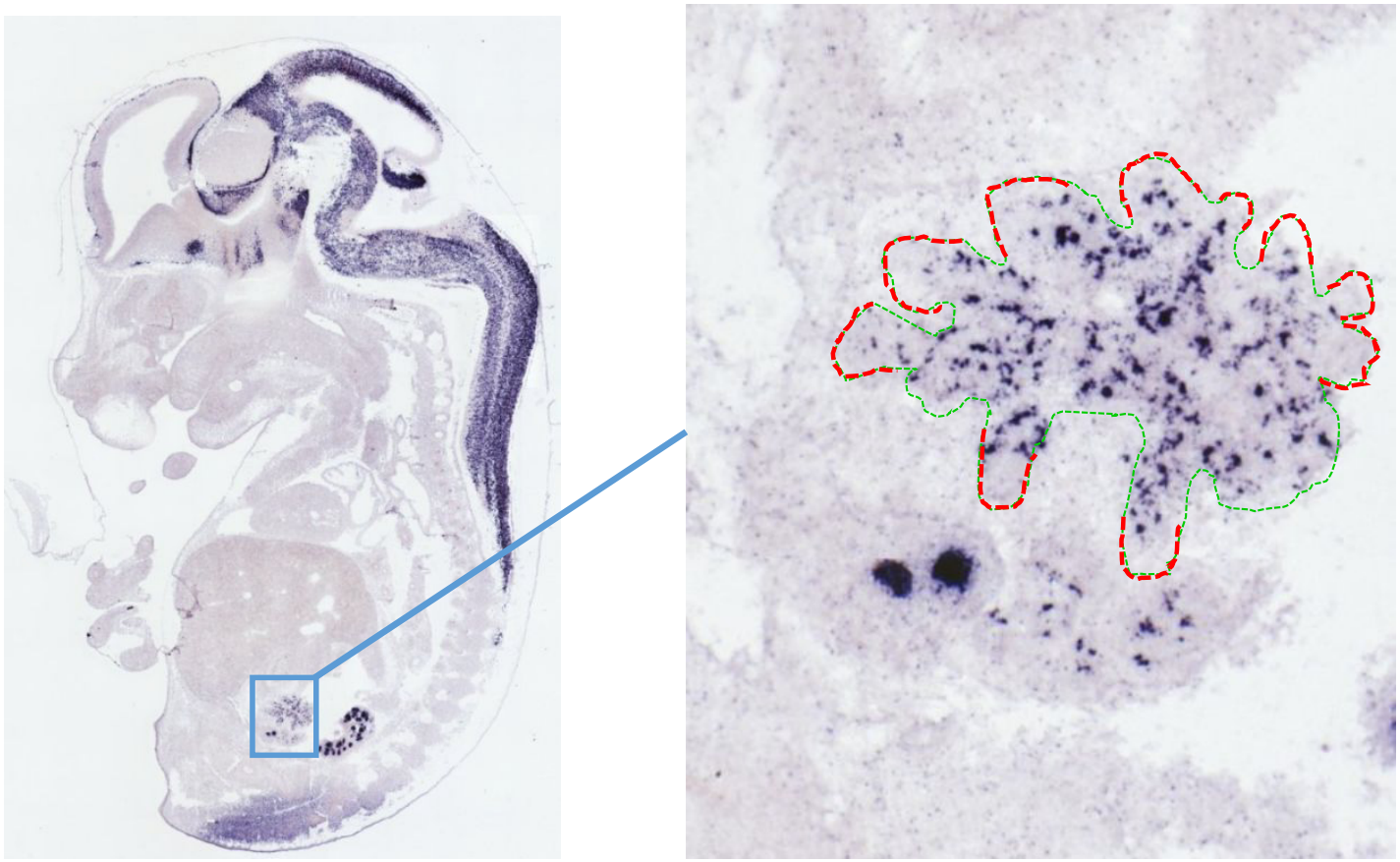


Figure 2.18—GenePaint Image Showing In Situ Hybridization with LHX1 probe [124]. The pancreas is outlined with tip regions in red and edges of trunk regions in green.

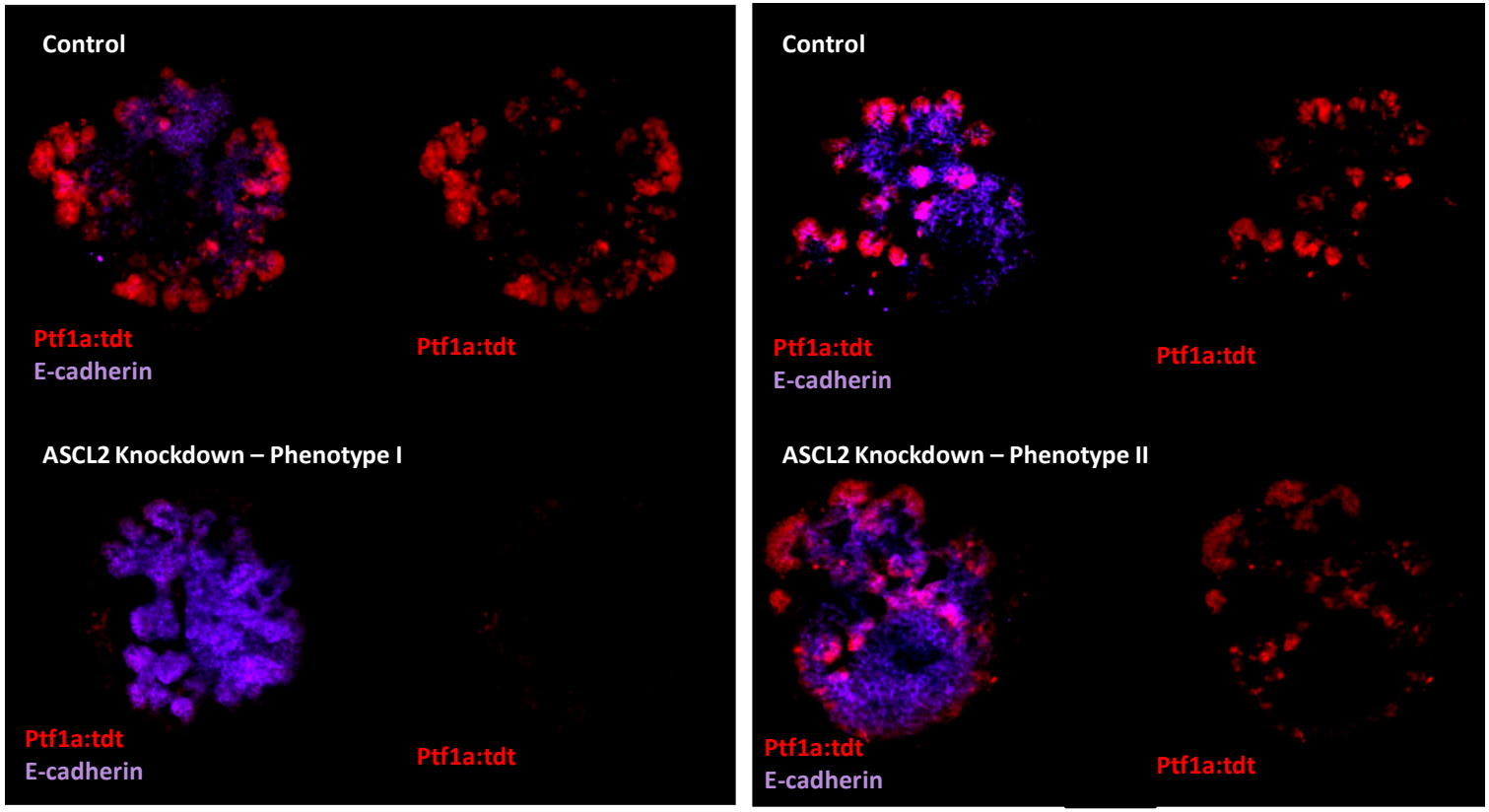
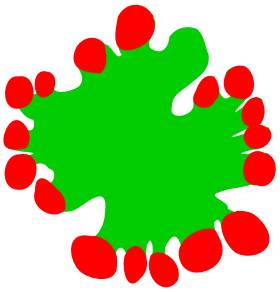
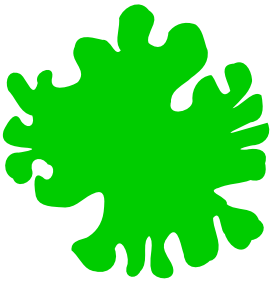
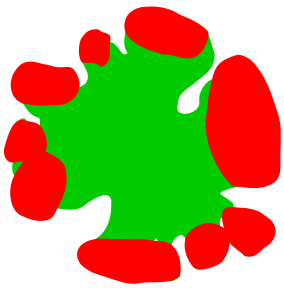


Figure 2.19—Confocal images of ASCL2 lentiviral shRNA knockdown phenotypes. In Phenotype I, there is no tdTomato signal present although the epithelium appears normal. In Phenotype II, there is tdTomato present, but the tips are dilated and have not bud off of the surrounding epithelium.

	Normal	Phenotype 1	Phenotype 2
Control	**12** 92%	**0** 0%	**1** 8%
ASCL2 KD	**5** 45%	**2** 18%	**4** 36%

Figure 2.20—Diagram and percentages of ASCL2 knockdown phenotypes. Of 13 control buds, 12 (92%) had a normal phenotype while 1 (8%) resembled phenotype 1. Of the 11 buds that were treated with Ascl2 lentiviral shRNA, 5 (45%) had a normal phenotype, while 2 (18%) were phenotype 1 and 4 (36%) were phenotype 2.

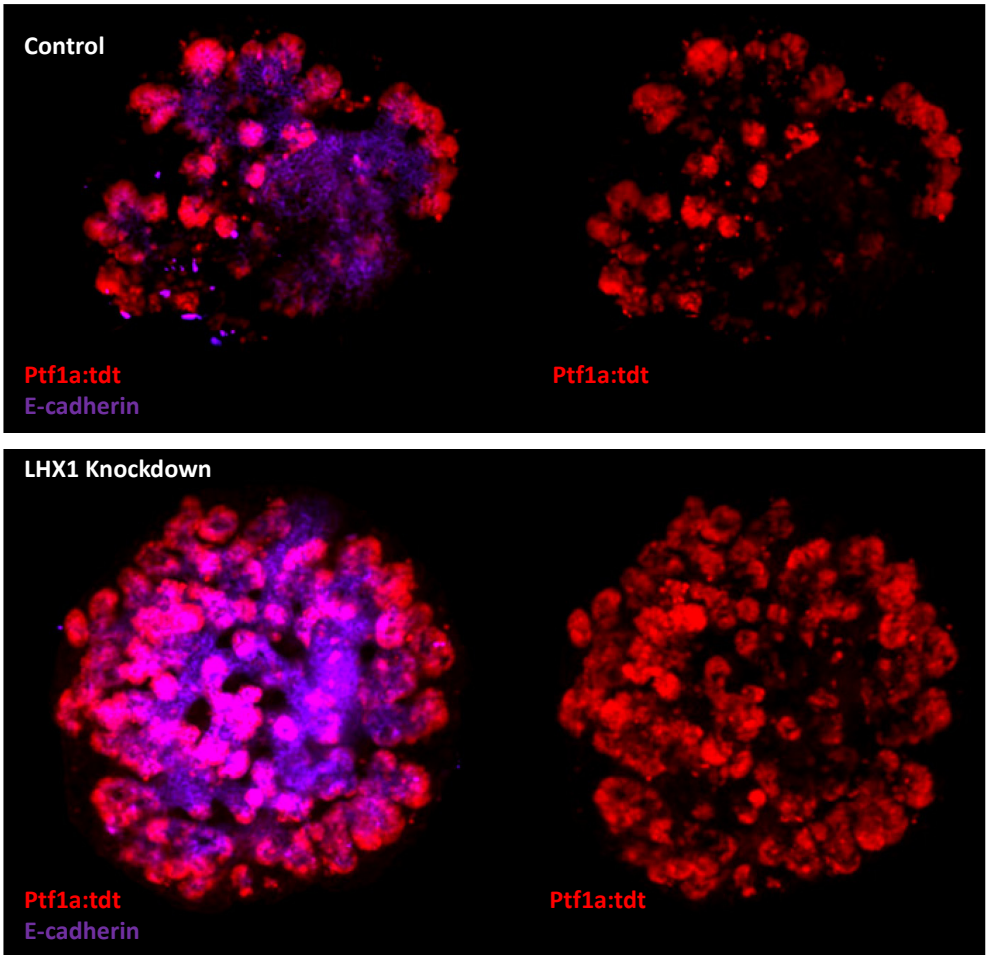
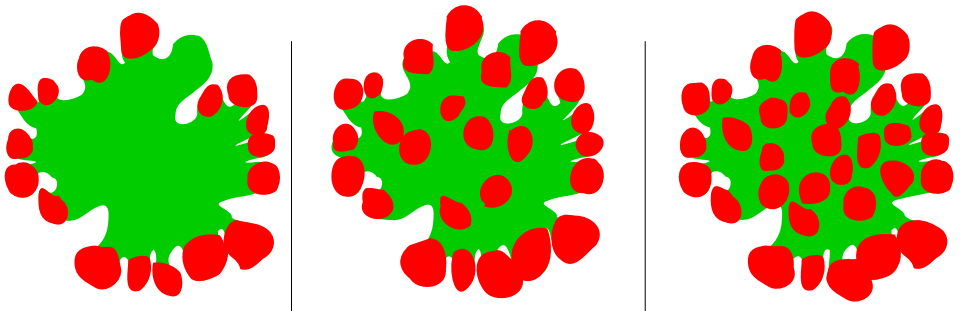


Figure 2.21—Confocal images of LHX1 lentiviral shRNA knockdown. Preliminary experiments indicate the treatment with LHX1 lentiviral shRNA causes an increase in the number of tips.



	17-24 tips	25-29 tips	>30 tips
Control	**6** 67%	**3** 33%	**0** 0%
LHX1 KD	**3** 38%	**3** 38%	**2** 25%

Figure 2.22—Diagram and percentages of LHX1 knockdown results. Initial experiments show an increase in the number of tips in dorsal buds treated with LHX1 lentiral shRNA. Of the 9 control buds, 6 (67%) had 17-24 tips, 3 (33%) had 25-29 tips and there were none with 30 or more tips. Of the 8 buds treated with Lhx1 lentiviral shRNA, 3 (38%) had 17-24 tips, 3 (38%) had 25-29 tips and 2 (25%) had 30 or more tips.

Table 2.1—All transcripts with standard deviation >6 above the mean expression level.

			E13.5 GFP vs. Double			
Transcript ID	Gene	RefSeq	Ratio	FC	Log2(FC)	SD
6927227	Fam213b	BC030453	1.94	1.94	0.96	+6s
6792260	BC006965	NR_024085	1.92	1.92	0.94	+6s
6832182	41885	NM_011889	1.97	1.97	0.98	+6s
6917554	Gm3579	AY140896	1.97	1.97	0.98	+6s
6770424	Gm6770	XM_003085723	1.90	1.90	0.93	+6s
7010644	Dock11	NM_001009947	1.94	1.94	0.95	+6s
7018773	Taf9b	NM_001001176	1.96	1.96	0.97	+6s
6924515	Elavl4	NM_010488	1.90	1.90	0.92	+6s
6748884	Il1r1	NM_008362	1.94	1.94	0.95	+6s
6860199	Pcdhb17	NM_053142	1.95	1.95	0.96	+6s
6759997	Scg2	NM_009129	1.98	1.98	0.99	+6s
6935348	Slc29a4	NM_146257	1.95	1.95	0.96	+6s
6940933	Slc26a1	NM_174870	1.94	1.94	0.95	+6s
7006739	Zfp808	NM_001039239	1.95	1.95	0.96	+6s
7009774	Syp	NM_009305	1.98	1.98	0.98	+6s
6926835	Gm13157	NM_001127189	1.94	1.94	0.96	+6s
6892699	Mafb	NM_010658	1.98	1.98	0.99	+6s
6917277	Fndc5	NM_027402	1.95	1.95	0.97	+6s
6781111	Slc36a1	NM_153139	1.93	1.93	0.95	+6s
6973206	Il11	NM_008350	1.94	1.94	0.96	+6s
6753700	B3galt2	NM_020025	1.92	1.92	0.94	+6s
6928972	Gnat3	NM_001081143	1.92	1.92	0.94	+6s
6762336	Gm19497	XR_107241	1.93	1.93	0.95	+6s
6748438	Khdrbs2	BC132117	1.98	1.98	0.98	+6s
6884444	A930010G16Rik	AK020843	1.97	1.97	0.98	+6s
7009927	Sytl5	NM_177704	1.96	1.96	0.97	+6s
6792418	Grin2c	NM_010350	1.98	1.98	0.99	+6s
6865025	Gm4013	NR_033452	1.98	1.98	0.99	+6s
6856892	2410021H03Rik	AK010563	1.92	1.92	0.94	+6s
6919417	Tox	NM_145711	1.92	1.92	0.94	+6s
6869646	Cc2d2b	XM_001000795	1.98	1.98	0.99	+6s
6884541	Gm10115	AK081831	1.93	1.93	0.95	+6s
6750143	Unc80	NM_175510	1.99	1.99	0.99	+6s
6900407	Wdr47	NM_181400	1.98	1.98	0.99	+6s
6867051	Galr1	NM_008082	1.94	1.94	0.96	+6s
7016297	Akap17b	NM_001081956	1.97	1.97	0.98	+6s
6992946	Entpd3	NM_178676	1.94	1.94	0.96	+6s
7002911	Rfc2	NM_020022	1.90	1.90	0.93	+6s
6869216	Papss2	NM_011864	1.99	1.99	0.99	+6s
6768340	D230019N24Rik	AK051927	1.90	1.90	0.92	+6s
7011837	Mamld1	NM_001081354	1.93	1.93	0.95	+6s

7011801	BC023829	NM_001033328	1.94	1.94	0.95	+6s
6801917	Rab15	NM_134050	1.93	1.93	0.95	+6s
6959575	Clip3	NM_001081114	2.30	2.30	1.20	+6s
6760754	Kif1a	NM_008440	2.02	2.02	1.02	+6s
7014538	A230072E10Rik	NR_015600	2.02	2.02	1.01	+6s
7009800	Pcsk1n	NM_013892	2.39	2.39	1.26	+6s
6791533	Ppy	NM_008918	2.42	2.42	1.28	+6s
7009135	4933409K07Rik	NR_033123	2.27	2.27	1.18	+6s
7018613	Dmrtc1a	NM_027591	2.33	2.33	1.22	+6s
6995208	Fxyd6	AK131913	2.38	2.38	1.25	+6s
6852130	Ypel5	NM_027166	2.42	2.42	1.27	+6s
6979514	Cotl1	AK196452	2.42	2.42	1.27	+6s
6871386	Gng3	NM_010316	2.64	2.64	1.40	+6s
6779818	Cpeb4	NM_026252	2.16	2.16	1.11	+6s
6815511	Cartpt	NM_013732	2.15	2.15	1.10	+6s
6973730	Lrrc8e	NM_028175	2.29	2.29	1.20	+6s
6749929	Zdbf2	NM_028673	2.03	2.03	1.02	+6s
6943841	Peg10	NM_130877	2.49	2.49	1.31	+6s
6972194	Sct	NM_011328	2.31	2.31	1.20	+6s
6796777	Gstz1	NM_010363	2.12	2.12	1.08	+6s
7015128	Gm19528	XR_106062	2.37	2.37	1.24	+6s
6913020	Npr2	NM_173788	2.30	2.30	1.20	+6s
6760917	Pam	NM_013626	2.08	2.08	1.05	+6s
6804900	Gng4	NM_010317	2.10	2.10	1.07	+6s
6815490	Map1b	NM_008634	2.02	2.02	1.02	+6s
6778719	Upp1	NM_009477	2.08	2.08	1.06	+6s
7012985	Pabpc1l2b-ps	XM_003085258	2.30	2.30	1.20	+6s
6768615	2310015B20Rik	ENSMUST00000020090	2.02	2.02	1.02	+6s
6785102	Gpr142	NM_181749	2.36	2.36	1.24	+6s
6963390	4930431P19Rik	AK031634	2.39	2.39	1.26	+6s
6917997	Camk2n1	NM_025451	2.05	2.05	1.03	+6s
6985363	Fa2h	NM_178086	2.76	2.76	1.46	+6s
6997349	Hmgn3	NM_026122	2.40	2.40	1.26	+6s
6888324	Serping1	NM_009776	2.46	2.46	1.30	+6s
6759264	Dytn	NM_001081658	2.54	2.54	1.35	+6s
6757637	Gm5699	ENSMUST00000152491	2.40	2.40	1.26	+6s
6781515	Mfap4	NM_029568	2.23	2.23	1.16	+6s
6762320	Atp2b4	NM_213616	2.50	2.50	1.32	+6s
7002049	Gm3579	AY140896	2.40	2.40	1.26	+6s
6824004	Cdhr1	NM_130878	2.29	2.29	1.19	+6s
7021194	Tspan7	NM_019634	2.01	2.01	1.01	+6s
6958835	Meis3	NM_008627	2.05	2.05	1.03	+6s
7015542	Sytl5	NM_177704	2.24	2.24	1.16	+6s

6912216	9330118A15Rik	AK033929	2.47	2.47	1.30	+6s
7012561	Pcyt1b	NM_211138	2.43	2.43	1.28	+6s
6830370	Slc30a8	NM_172816	2.76	2.76	1.46	+6s
6933072	Spp1	NM_009263	2.15	2.15	1.10	+6s
7014982	Ap1s2	NM_026887	2.60	2.60	1.38	+6s
6870925	Gal	NM_010253	2.28	2.28	1.19	+6s
6770876	Gm10752	ENSMUST00000099268	2.15	2.15	1.10	+6s
6975614	Vps37a	NM_033560	2.14	2.14	1.10	+6s
6965078	Prap1	NM_009475	2.16	2.16	1.11	+6s
6902231	Ttll7	NM_027594	2.32	2.32	1.21	+6s
7020314	Kctd12b	NM_175429	2.71	2.71	1.44	+6s
6943974	Tac1	NM_009311	2.29	2.29	1.19	+6s
6749933	Adam23	NM_001177600	2.38	2.38	1.25	+6s
6763247	Fam163a	NM_177838	2.17	2.17	1.11	+6s
7009834	Slc38a5	NM_172479	2.50	2.50	1.32	+6s
6839631	Efcab1	NM_025769	2.32	2.32	1.21	+6s
6754138	Rgs16	NM_011267	2.37	2.37	1.24	+6s
6885912	Stxbp1	NM_001113569	2.43	2.43	1.28	+6s
6864518	Nme5	NM_080637	2.60	2.60	1.38	+6s
6791526	Dusp3	ENSMUST00000003612	2.77	2.77	1.47	+6s
7016666	Smarca1	NM_053123	2.23	2.23	1.16	+6s
6778052	Suox	NM_173733	2.37	2.37	1.25	+6s
6934854	Gatsl2	NM_030719	2.10	2.10	1.07	+6s
6887187	Gcg	NM_008100	2.61	2.61	1.38	+6s
7013015	Chic1	NM_009767	2.05	2.05	1.04	+6s
6805468	Dcdc2a	NM_177577	2.07	2.07	1.05	+6s
6788723	Rasd1	NM_009026	1.99	1.99	0.99	+6s
6783809	Gip	NM_008119	2.10	2.10	1.07	+6s
6982918	Cpe	NM_013494	2.79	2.79	1.48	+6s
6994583	9330161A08Rik	AK048946	2.40	2.40	1.26	+6s
6990178	9530091C08Rik	NR_033299	2.62	2.62	1.39	+6s
6870375	Ins1	NM_008386	2.46	2.46	1.30	+6s
6755643	9330156P08Rik	AK050330	2.10	2.10	1.07	+6s
7014048	Zcchc18	NM_001035510	2.74	2.74	1.45	+6s
6973542	Trim12c	NM_001146007	2.66	2.66	1.41	+6s
6967799	A330076H08Rik	NR_015599	2.25	2.25	1.17	+6s
6814206	Adamts16	NM_172053	2.36	2.36	1.24	+6s
6838996	Zfp174	NM_001081217	2.10	2.10	1.07	+6s
6854415	Baiap3	NM_001163270	2.35	2.35	1.23	+6s
6845978	Cd200	NM_010818	2.06	2.06	1.05	+6s
6942841	Mmd2	NM_175217	2.00	2.00	1.00	+6s
6967006	Sult2b1	NM_017465	2.00	2.00	1.00	+6s
6899589	Celf3	NM_172434	2.59	2.59	1.37	+6s

6994581	9330161A08Rik	AK048946	2.32	2.32	1.21	+6s
6789084	Dnah9	NM_001099633	2.14	2.14	1.10	+6s
6927510	Gm3831	AK156722	2.23	2.23	1.16	+6s
6775203	Gm10941	NR_026944	2.14	2.14	1.10	+6s
7009923	Sytl5	NM_177704	2.58	2.58	1.37	+6s
7020624	Reps2	NM_178256	2.08	2.08	1.06	+6s
6792255	BC006965	NR_024085	2.19	2.19	1.13	+6s
6934766	4930563F08Rik	AK016203	2.48	2.48	1.31	+6s
6990435	Rab27a	NM_023635	2.06	2.06	1.04	+6s
6825705	Sftpc	NM_011359	2.43	2.43	1.28	+6s
6778298	Selm	NM_053267	2.39	2.39	1.26	+6s
6747354	St18	NM_173868	2.62	2.62	1.39	+6s
6933731	Srrm4os	NR_015595	2.40	2.40	1.26	+6s
6983056	Psd3	NM_030263	2.45	2.45	1.29	+6s
6766579	Vnn1	NM_011704	2.05	2.05	1.03	+6s
6894394	Gjd2	NM_010290	2.67	2.67	1.41	+6s
6759764	Fev	NM_153111	2.25	2.25	1.17	+6s
6856512	Fbxl17	NM_015794	2.12	2.12	1.08	+6s
6887661	5830411J07Rik	AK017919	2.02	2.02	1.01	+6s
6928275	Gm12888	NM_001033791	2.66	2.66	1.41	+6s
6994133	Glb1l2	NM_153803	2.10	2.10	1.07	+6s
6791007	Gm20332	XR_106395	2.09	2.09	1.06	+6s
6880467	Disp2	NM_170593	1.99	1.99	1.00	+6s
6904545	1700034I23Rik	BC117739	2.09	2.09	1.06	+6s
6881488	Snap25	NM_011428	2.34	2.34	1.23	+6s
6907089	Riiad1	NM_025506	2.10	2.10	1.07	+6s
6883109	Wfdc10	NM_001039501	2.70	2.70	1.44	+6s
6750434	Smarcal1	NM_018817	2.14	2.14	1.10	+6s
6992362	Gm10621	ENSMUST00000098384	2.07	2.07	1.05	+6s
6808279	Pcsk1	NM_013628	2.75	2.75	1.46	+6s
6824321	Gch1	NM_008102	2.59	2.59	1.37	+6s
6925547	Hpca	NM_001130419	2.75	2.75	1.46	+6s
6843333	Gm9510	AK043809	2.52	2.52	1.34	+6s
6954385	Gadd45a	NM_007836	2.34	2.34	1.23	+6s
6971312	Asphd1	NM_001039645	2.01	2.01	1.01	+6s
6983771	Tbc1d9	NM_001111304	2.05	2.05	1.04	+6s
6755896	Dusp10	NM_022019	2.06	2.06	1.05	+6s
7014176	Atg4a	NM_174875	2.34	2.34	1.23	+6s
6845059	C330004M20Rik	AK021176	2.27	2.27	1.18	+6s
6921331	Igfbpl1	NM_018741	2.02	2.02	1.02	+6s
6894617	Ctcfl	NM_001081387	2.21	2.21	1.15	+6s
6750390	Gm5528	ENSMUST00000072765	2.27	2.27	1.18	+6s
6889440	A130004G07Rik	AK037298	2.60	2.60	1.38	+6s

6994582	9330161A08Rik	AK079083	2.45	2.45	1.29	+6s
6815491	5330431K02Rik	AK019926	2.38	2.38	1.25	+6s
6947581	4930553P18Rik	AK016112	2.03	2.03	1.02	+6s
6748525	Dst	NM_134448	2.11	2.11	1.08	+6s
6899562	C2cd4d	NM_001136117	2.01	2.01	1.01	+6s
6986987	Phxr4	NR_028271	2.55	2.55	1.35	+6s
6924583	Slc5a9	NM_145551	2.29	2.29	1.19	+6s
6889898	Gjd2	NM_010290	2.55	2.55	1.35	+6s
6872749	Lipo1	NM_001013770	2.14	2.14	1.10	+6s
6864753	Pcdhga12	AK036054	2.00	2.00	1.00	+6s
7014268	Pak3	NM_001195049	2.28	2.28	1.19	+6s
6970001	Trim12c	NM_001146007	2.60	2.60	1.38	+6s
6838303	Amigo2	NM_178114	2.44	2.44	1.28	+6s
7008144	Otop1	NM_172709	2.53	2.53	1.34	+6s
6750148	Unc80	NM_175510	2.19	2.19	1.13	+6s
7023092	Rbmy	NM_011253	2.08	2.08	1.05	+6s
6927968	Cdc26	AK028893	2.32	2.32	1.22	+6s
6962740	8030425K09Rik	AK020195	2.26	2.26	1.17	+6s
7011791	1110012L19Rik	BC024574	2.01	2.01	1.01	+6s
6747450	A130040M12Rik	NR_002860	2.06	2.06	1.05	+6s
6848822	Ermard	AK019108	2.00	2.00	1.00	+6s
6802014	4633402D09Rik	AK014620	2.28	2.28	1.19	+6s
7009603	Dnah7b	NM_001160386	2.42	2.42	1.28	+6s
6868909	C030016D13Rik	NR_027987	2.36	2.36	1.24	+6s
6960359	Mtag2	NR_027802	2.13	2.13	1.09	+6s
6965758	Gipr	NM_001080815	2.09	2.09	1.06	+6s
6960444	Ush1c	NM_153677	2.21	2.21	1.15	+6s
6856542	4930405O22Rik	AK160416	2.23	2.23	1.16	+6s
6953887	Pde1c	NM_001025568	2.44	2.44	1.28	+6s
6761138	Rnf152	NM_178779	2.53	2.53	1.34	+6s
7024480	Rbmy	NM_011253	2.51	2.51	1.33	+6s
6789789	Rph3al	NM_029548	2.61	2.61	1.38	+6s
6850063	Fkbpl	NM_019873	2.00	2.00	1.00	+6s
6790340	Lhx1	NM_008498	2.64	2.64	1.40	+6s
7008453	C87414	NM_001164284	2.09	2.09	1.06	+6s
6967027	Abcc8	NM_011510	2.02	2.02	1.01	+6s
6984156	Tox3	NM_172913	2.69	2.69	1.43	+6s
6994580	LOC100503226	XR_107257	2.61	2.61	1.39	+6s
6749537	4930444A19Rik	AK015376	2.31	2.31	1.21	+6s
6837280	Dnajb7	NM_021317	1.99	1.99	0.99	+6s
6785079	Ttyh2	NM_053273	2.10	2.10	1.07	+6s
6834108	Cdh6	NM_007666	2.35	2.35	1.23	+6s
6772476	Slc35d3	NM_029529	2.05	2.05	1.04	+6s

6860202	Pcdhb18	NM_053143	2.36	2.36	1.24	+6s
6808399	C130051F05Rik	AK037553	2.09	2.09	1.06	+6s
6813849	A930032L01Rik	AK020918	2.06	2.06	1.04	+6s
6906976	Lce6a	NM_001166172	2.16	2.16	1.11	+6s
6782273	Atp2a3	NM_016745	2.18	2.18	1.13	+6s
6898282	A330078L11Rik	AK039653	2.47	2.47	1.30	+6s
6758734	Ankrd44	NM_001081433	2.02	2.02	1.02	+6s
6757770	Dst	NM_133833	2.30	2.30	1.20	+6s
6915993	Dab1	NM_177259	2.41	2.41	1.27	+6s
7023088	Rbmy	NM_011253	2.72	2.72	1.44	+6s
6759653	41702	NM_001045533	2.07	2.07	1.05	+6s
6977751	Cacna1a	NM_007578	2.57	2.57	1.36	+6s
6972130	Olfr532	NM_147026	2.31	2.31	1.21	+6s
6817580	A930006J02Rik	AK076864	2.01	2.01	1.01	+6s
7024486	Rbmy	NM_011253	2.39	2.39	1.26	+6s
7017419	Ids	NM_010498	3.39	3.39	1.76	+6s
6879161	Syt13	NM_030725	2.95	2.95	1.56	+6s
6823284	Kcnma1	NM_010610	2.82	2.82	1.50	+6s
7015001	Tmem27	NM_020626	3.41	3.41	1.77	+6s
6903983	Slc7a14	NM_172861	3.60	3.60	1.85	+6s
6759792	Resp18	NM_009049	2.81	2.81	1.49	+6s
6828379	Plcxd3	NM_177355	3.09	3.09	1.63	+6s
6934855	Gatsl2	ENSMUST00000016088	2.84	2.84	1.51	+6s
6854787	Tff3	NM_011575	3.10	3.10	1.63	+6s
6999522	Cck	NM_031161	2.89	2.89	1.53	+6s
6883013	Gdap1l1	NM_144891	2.95	2.95	1.56	+6s
6950750	Iapp	NM_010491	3.56	3.56	1.83	+6s
6791534	Pyy	NM_145435	2.96	2.96	1.57	+6s
6911833	Runx1t1	NM_001111027	2.91	2.91	1.54	+6s
6764797	1700056E22Rik	NM_028516	3.35	3.35	1.74	+6s
6788627	Fam183b	NM_029283	3.63	3.63	1.86	+6s
6943605	Mkrn1	NM_018810	2.88	2.88	1.53	+6s
6949335	Cxcl12	NM_001012477	3.11	3.11	1.64	+6s
6978263	Gnao1	NM_010308	3.56	3.56	1.83	+6s
6996956	Scg3	NM_009130	2.88	2.88	1.53	+6s
6951282	Hepacam2	NM_178899	3.29	3.29	1.72	+6s
6894718	Gdap1l1	NM_144891	3.14	3.14	1.65	+6s
6837045	Mfng	NM_008595	2.85	2.85	1.51	+6s
6925361	Tekt2	NM_011902	3.19	3.19	1.67	+6s
7019656	Tmsb15l	NM_207267	3.35	3.35	1.74	+6s
6890368	Tgm7	NM_001160424	3.07	3.07	1.62	+6s
6860201	Pcdhb18	NM_053143	2.93	2.93	1.55	+6s
6760251	Sphkap	NM_172430	2.80	2.80	1.49	+6s

6942121	Rimbp2	NM_001081388	3.11	3.11	1.64	+6s
6758668	Dnah7b	NM_001160386	2.94	2.94	1.56	+6s
6839943	Vwa5b2	NM_001144953	3.63	3.63	1.86	+6s
6845980	Gm609	NM_001005854	3.32	3.32	1.73	+6s
6845987	Gm609	NM_001005854	3.08	3.08	1.62	+6s
6972319	Ins2	NM_001185084	3.29	3.29	1.72	+6s
7014837	Map3k15	NM_001163085	3.11	3.11	1.64	+6s
6764120	Nhlh1	NM_010916	3.37	3.37	1.75	+6s
6885938	Lmx1b	ENSMUST00000041730	2.86	2.86	1.51	+6s
6747766	Kcnb2	NM_001098528	2.92	2.92	1.54	+6s
6802511	Gm6566	BC042778	3.12	3.12	1.64	+6s
6961280	D930030O05Rik	AK083489	2.80	2.80	1.49	+6s
7015946	6330509M05Rik	AK031957	3.41	3.41	1.77	+6s
6791564	Rundc3a	AK007019	3.10	3.10	1.63	+6s
6838338	Vdr	NM_009504	2.99	2.99	1.58	+6s
6798404	Ptprn2	NM_011215	2.91	2.91	1.54	+6s
6864527	Gfra3	NM_010280	2.82	2.82	1.49	+6s
6868360	LOC553096	AK047890	2.82	2.82	1.50	+6s
6962951	D930046H04Rik	AK048487	3.02	3.02	1.59	+6s
6810359	Gm10033	XR_105228	2.81	2.81	1.49	+6s
6784588	Kcnh6	NM_001037712	3.55	3.55	1.83	+6s
6810118	9530083O12Rik	AK079287	3.43	3.43	1.78	+6s
6783388	4931406E20Rik	AK016433	2.86	2.86	1.52	+6s
6912883	Spink4	NM_011463	2.81	2.81	1.49	+6s
6798034	Mir300	NR_029651	0.26	-3.81	-1.93	= -6s
7013294	Pou3f4	NM_008901	4.14	4.14	2.05	> +6s
6825573	Nefm	NM_008691	3.84	3.84	1.94	> +6s
6816288	Isl1	NM_021459	3.89	3.89	1.96	> +6s
6910307	Gm20292	XR_107565	6.61	6.61	2.73	> +6s
6956581	Ghrl	NM_021488	4.30	4.30	2.10	> +6s
7005290	Spock1	NM_009262	3.96	3.96	1.99	> +6s
6928909	Cacna2d1	NM_001110843	4.12	4.12	2.04	> +6s
6881771	Pcsk2	NM_008792	4.56	4.56	2.19	> +6s
6794338	Efcab10	NM_029152	4.84	4.84	2.27	> +6s
6797496	Chga	NM_007693	3.88	3.88	1.96	> +6s
6767797	Rfx6	NM_001159389	6.38	6.38	2.67	> +6s
6888114	Neurod1	NM_010894	5.55	5.55	2.47	> +6s
6844649	Sst	NM_009215	4.77	4.77	2.25	> +6s
6796060	Dbpht2	NM_198866	4.67	4.67	2.22	> +6s
6812771	Gfod1	NM_001033399	4.33	4.33	2.11	> +6s
6796059	1700086L19Rik	NR_030733	6.71	6.71	2.75	> +6s
6881337	Chgb	NM_007694	4.43	4.43	2.15	> +6s
6917157	Tekt2	NM_011902	5.05	5.05	2.34	> +6s

6879741	Pax6	NM_013627	3.90	3.90	1.97	> +6s
6768266	Neurog3	NM_009719	5.13	5.13	2.36	> +6s
6822731	Cadps	NM_012061	5.63	5.63	2.49	> +6s
6772079	Adgb	ENSMUST00000148816	4.52	4.52	2.18	> +6s
6759765	Cryba2	NM_021541	3.67	3.67	1.88	> +6s
6845459	Stxbp5l	NM_172440	3.75	3.75	1.91	> +6s
6952294	Pax4	NM_011038	4.45	4.45	2.15	> +6s
6783818	Ttll6	NM_172799	3.66	3.66	1.87	> +6s
6898277	A330078L11Rik	AK039653	4.72	4.72	2.24	> +6s
6941118	Miat	NR_033657	4.63	4.63	2.21	> +6s
6749935	Adam23	NM_011780	4.89	4.89	2.29	> +6s
6889426	Gm20279	XR_107384	5.38	5.38	2.43	> +6s
6928620	Gm8773	NR_033499	3.93	3.93	1.97	> +6s
6756189	Ush2a	NM_021408	3.74	3.74	1.90	> +6s
6845455	Stxbp5l	NM_001114612	4.15	4.15	2.05	> +6s
6884296	Myt1	NM_008665	3.96	3.96	1.98	> +6s
6982461	C130073E24Rik	AK045360	4.23	4.23	2.08	> +6s
6811716	Scgn	NM_145399	5.63	5.63	2.49	> +6s
6922886	Cer1	NM_009887	4.36	4.36	2.12	> +6s
6839944	Vwa5b2	NM_001144953	3.65	3.65	1.87	> +6s
6827452	Slitrk6	NM_175499	10.49	10.49	3.39	> +6s
6781970	Aurkb	NM_011496	0.52	-1.94	-0.96	-6s
6945109	Cpa2	NM_001024698	0.50	-2.00	-1.00	-6s
6935439	Bhlha15	NM_010800	0.46	-2.17	-1.12	-6s
6870745	Pnliprp1	NM_018874	0.39	-2.59	-1.37	-6s
6908330	Amy2a5	NM_001042711	0.46	-2.19	-1.13	-6s
6908345	Amy2a5	NM_001042711	0.49	-2.05	-1.03	-6s
6908348	Amy2a5	NM_001042711	0.51	-1.94	-0.96	-6s
6938259	Cckar	NM_009827	0.48	-2.07	-1.05	-6s
6908347	Amy2a5	NM_001042711	0.48	-2.08	-1.06	-6s
6908332	Amy2a5	NM_001042711	0.50	-1.99	-1.00	-6s
6908077	Gstm3	NM_010359	0.46	-2.18	-1.13	-6s
6845551	Pla1a	NM_134102	0.42	-2.36	-1.24	-6s
6885360	Noxa1	NM_172204	0.43	-2.32	-1.22	-6s
6913011	Car9	NM_139305	0.40	-2.51	-1.33	-6s
6953089	Try5	NM_001003405	0.49	-2.04	-1.03	-6s
6985087	Tmed6	NM_025458	0.40	-2.51	-1.33	-6s
6983639	Hhip	NM_020259	0.42	-2.36	-1.24	-6s
6989222	Crabp1	NM_013496	0.40	-2.50	-1.32	-6s
6907648	Mab21l3	NM_172295	0.45	-2.25	-1.17	-6s
6794513	Sostdc1	NM_025312	0.44	-2.29	-1.19	-6s
6917774	Il22ra1	NM_178257	0.41	-2.46	-1.30	-6s
6959586	Nphs1	NM_019459	0.51	-1.98	-0.99	-6s

6997034	Tinag	NM_012033	0.45	-2.22	-1.15	-6s
6854826	Cbs	NM_144855	0.47	-2.11	-1.08	-6s
6775838	Dram1	NM_027878	0.45	-2.20	-1.14	-6s
6843315	1810044K17Rik	AK007778	0.43	-2.31	-1.21	-6s
7003348	Gm1604b	NM_001033442	0.48	-2.08	-1.05	-6s
6910960	Lrrc7	NM_001081358	0.51	-1.95	-0.96	-6s
7000914	Vmn2r57	NM_177764	0.43	-2.31	-1.21	-6s
6947176	Reg1	NM_009042	0.49	-2.03	-1.02	-6s
6973041	Ceacam16	NM_001033419	0.42	-2.36	-1.24	-6s
6977068	Ankle1	NM_172756	0.51	-1.95	-0.96	-6s
6906086	Serpini2	NM_026460	0.40	-2.52	-1.33	-6s
6972329	Ascl2	NM_008554	0.46	-2.16	-1.11	-6s
6970009	Hbb-y	NM_008221	0.49	-2.05	-1.03	-6s
6892903	Matn4	NM_013592	0.41	-2.42	-1.27	-6s
6917273	Tmem54	NM_025452	0.38	-2.61	-1.38	-6s
6756345	Nsl1	NM_198654	0.51	-1.97	-0.98	-6s
6873241	Cpn1	NM_030703	0.47	-2.15	-1.10	-6s
6779995	Slit3	NM_011412	0.44	-2.28	-1.19	-6s
6824932	Gjb2	NM_008125	0.39	-2.59	-1.38	-6s
7019800	Nup62cl	NM_001081668	0.44	-2.26	-1.17	-6s
6751709	Bok	NM_016778	0.48	-2.08	-1.06	-6s
6825305	Gata4	NM_008092	0.41	-2.46	-1.30	-6s
6966286	Nphs1os	NR_004443	0.38	-2.65	-1.41	-6s
6782702	Vtn	NM_011707	0.46	-2.19	-1.13	-6s
6973004	Zfp296	NM_022409	0.41	-2.43	-1.28	-6s
6830927	Myc	NM_010849	0.41	-2.45	-1.29	-6s
6936589	Reln	NM_011261	0.40	-2.48	-1.31	-6s
6782705	Poldip2	AK169503	0.38	-2.64	-1.40	-6s
6818498	Mat1a	NM_133653	0.50	-2.02	-1.01	-6s
6921290	Shb	ENSMUST00000098095	0.51	-1.94	-0.96	-6s
6789908	Pipox	NM_008952	0.45	-2.24	-1.17	-6s
6945704	Prss2	NM_009430	0.47	-2.13	-1.09	-6s
6964247	Tbx6	NM_011538	0.50	-2.02	-1.01	-6s
6844558	Etv5	NM_023794	0.46	-2.20	-1.13	-6s
6945715	Gm5771	NM_001038997	0.49	-2.04	-1.03	-6s
6791519	Etv4	NM_008815	0.51	-1.98	-0.98	-6s
6938171	Lgi2	NM_144945	0.51	-1.98	-0.98	-6s
6750017	BC019684	BC040095	0.49	-2.06	-1.04	-6s
6900180	Slc16a1	NM_009196	0.51	-1.96	-0.97	-6s
6980845	1700014L14Rik	AK005981	0.50	-2.01	-1.01	-6s
6884646	Itih2	NM_010582	0.40	-2.50	-1.32	-6s
6848707	Plg	NM_008877	0.42	-2.36	-1.24	-6s
6956507	Srgap3	NM_080448	0.51	-1.96	-0.97	-6s

6932372	Cxcl15	NM_011339	0.48	-2.08	-1.06	-6s
6891336	Jag1	NM_013822	0.46	-2.19	-1.13	-6s
6799852	Dus4l	NM_028002	0.49	-2.04	-1.03	-6s
6768031	Gm9956	ENSMUST00000067972	0.46	-2.16	-1.11	-6s
6917040	Mir697	NR_030479	0.45	-2.21	-1.14	-6s
6805291	Vmn1r-ps103	NM_134211	0.51	-1.95	-0.96	-6s
6816861	Mnd1-ps	NR_030680	0.47	-2.15	-1.10	-6s
6881794	Banf2	NM_207275	0.47	-2.14	-1.10	-6s
6910956	B230334C09Rik	AK046021	0.40	-2.48	-1.31	-6s
6791309	Tns4	NM_172564	0.51	-1.95	-0.97	-6s
6907221	Ciart	BC132471	0.51	-1.97	-0.98	-6s
6854478	Hba-ps4	XM_356935	0.49	-2.04	-1.03	-6s
6807316	Gm3045	XM_003085857	0.49	-2.05	-1.04	-6s
6963156	Olfr661	NM_146748	0.42	-2.38	-1.25	-6s
6891494	Flrt3	AK045146	0.47	-2.12	-1.08	-6s
7018582	Ercc6l	NM_146235	0.51	-1.98	-0.98	-6s
6785463	Npb	NM_153288	0.48	-2.07	-1.05	-6s
6798016	Mir411	NR_029916	0.41	-2.47	-1.30	-6s
6901456	Gm5549	XM_485313	0.44	-2.26	-1.18	-6s
6811518	Olfr1362	NM_146744	0.50	-1.99	-0.99	-6s
6786473	Slc1a4	NM_018861	0.51	-1.98	-0.98	-6s
6844250	Mir130b	NR_029659	0.49	-2.05	-1.04	-6s
6981091	1810011O10Rik	NM_026931	0.48	-2.09	-1.06	-6s
6960299	Nup62-il4i1	NM_001171024	0.42	-2.40	-1.26	-6s
6919190	Mxra8	NM_024263	0.48	-2.07	-1.05	-6s
6781474	Dhrs7b	ENSMUST00000108718	0.45	-2.23	-1.16	-6s
6751292	Rpl30-ps6	ENSMUST00000086964	0.46	-2.17	-1.12	-6s
6934408	Tmem132b	NM_001190352	0.51	-1.96	-0.97	-6s
6780842	Olfr54	NM_010997	0.49	-2.03	-1.02	-6s
6890835	Ckap2l	NM_181589	0.50	-1.99	-0.99	-6s
6973229	Dnaaf3	BC150983	0.47	-2.12	-1.08	-6s
7002235	Aida	AK052622	0.49	-2.03	-1.02	-6s
6771832	Gm3213	XM_003085669	0.45	-2.20	-1.14	-6s
7013879	Gm20320	XR_108218	0.47	-2.13	-1.09	-6s
6885624	Cel	NM_009885	0.33	-3.00	-1.58	-6s
6908340	Amy2a5	NM_001042711	0.30	-3.36	-1.75	-6s
6883094	Rbpjl	NM_009036	0.36	-2.75	-1.46	-6s
6838578	Cela1	NM_033612	0.34	-2.90	-1.54	-6s
6870746	Pnliprp2	NM_011128	0.33	-3.07	-1.62	-6s
6894907	Camk1d	NM_177343	0.29	-3.41	-1.77	-6s
6875423	Ptf1a	NM_018809	0.29	-3.43	-1.78	-6s
6998643	Slc38a3	NM_023805	0.36	-2.77	-1.47	-6s
6837897	Cpne8	NM_025815	0.36	-2.76	-1.46	-6s

7000449	Prss3	NM_011645	0.32	-3.13	-1.65	-6s
6935475	Trrap	AK135094	0.29	-3.40	-1.76	-6s
6907224	Car14	NM_011797	0.36	-2.81	-1.49	-6s
6768829	Pcdh15	NM_001142746	0.34	-2.91	-1.54	-6s
6952655	Tmem140	NM_197986	0.35	-2.88	-1.53	-6s
6790237	1190001M18Rik	BC023846	0.37	-2.72	-1.45	-6s
6838954	Fam83a	NM_173862	0.35	-2.87	-1.52	-6s
6765422	Gm6403	AK032775	0.30	-3.35	-1.74	-6s
6832390	Ppara	NM_011144	0.36	-2.78	-1.47	-6s
6817951	Sfmbt1	NM_001166532	0.36	-2.78	-1.48	-6s

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Publications

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